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TISSUE SPECIFIC ADENOVIRAL VECTORS

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of application serial no. 08/699,753, filed June 26, 1996, which is a continuation-in-part of application serial no. 08/495,034, filed June 27, 1995, the disclosure of which is herein incorporated by reference. This application is also a continuation-in part of application serial number 09/033,428, filed March 2, 1998, which claims the benefit of provisional application serial number 60/039,597, filed March 3, 1998; and a continuation-in-part of application serial number 09/033,555, filed March 2, 1998, which claims the benefit of provisional application serial number 60/039,763 filed March 3, 1997; and a continuation-in-part of application serial number 09/033,333, filed March 2, 1998, which claims the benefit of provisional application serial number 60/039,762, filed March 3, 1997. This application also claims the benefit of application serial number 60/039,599, filed March 3, 1997. All of the above patent applications are incorporated by reference herein.

STATEMENT OF RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH

(Not Applicable)

TECHNICAL FIELD

The field of this invention is cell transfection, particularly by adenoviral vectors.

BACKGROUND

The ability to change the genotype and phenotype of cells *in vitro* and *in vivo* has many applications. For studying physiologic processes, particularly with dedicated cells, there is substantial interest in being able to modify the phenotype to affect a particular process. By enhancing or depressing the amount of a member of the physiological pathway, by inhibiting the activity of a member of the pathway, by providing an allele or mutated analog of the naturally occurring member, one may be able to unravel the role of the various members in the pathway, the order in which the members participate, the presence of alternative pathways and the like. Also, one can use the cells for producing proteins.

Adenovirus does not require cell proliferation for efficient transduction of cells. Adenovirus modified by introduction of a transgene provides for transient expression of proteins. Adenovirus can be rendered incompetent by inactivating one or more essential genes and then be packaged in a helper cell line for use in transfection. Thus, adenovirus affords a convenient vehicle for modifying cellular traits or killing cells, as appropriate.

For many medical applications, there is an interest in being able to specifically modify target cells *in vivo* or *ex vivo*. The modification can be associated with random DNA integration, whereby a genetic capability is introduced that complements a genetic defect intracellularly, provides for secretion of a product from the modified cells, which is otherwise indetectably produced or not produced by the host, provide protection from disease, particularly viral disease, and the like. In many situations, in order to be effective, one must have a high efficiency of transfection of the target cells. This is particularly true for *in vivo* modification. In addition, one would wish to have a high specificity for the target cells, as compared to other cells that may be present *ex vivo* or *in vivo*.

Gene therapy involves the transfer of cloned genes to target cells. A variety of viral and non-viral vehicles have been developed to transfer these genes. Of the viruses, retroviruses, herpes virus, adeno-associated virus, Sindbis virus, poxvirus and adenoviruses have been used for gene transfer. These vehicles all have different properties. For example, retroviruses transduce genes *in vitro* with high efficiency by integrating the transduced gene into the chromosome following division of infected cells. Adeno-associated viruses can stabily integrate into and express transduced genes in both dividing and quiescent cells. In contrast, liposomes and adenovirus allow only transient gene expression, and transduce both dividing and quiescent target cells.

Of the viruses, adenoviruses are among the most easily produced and purified, whereas retroviruses are unstable, difficult to produce and impossible to purify. Both classes of virus transduce cells with high efficiency. Liposomes hold the promise of allowing repeat doses of genes for, unlike viruses, they are not immunogenetic. However, liposomes completed with DNA are difficult to produce in commercial quantities, and are inefficient gene transfer vehicles, most often transducing fewer than one percent of target cells.

Publications describing various aspects of adenovirus biology and/or techniques relating to adenovirus include the following. Graham and Van de Eb (1973) *Virology* 52:456-467; Takiff et al. (1981) *Lancet* ii:832-834; Berkner and Sharp (1983) *Nucleic Acid Research* 6003-6020; Graham (1984) *EMBO J* 3:2917-2922; Bett et al. (1993) *J. Virology* 67:5911-5921; and Bett et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:8802-8806 describe adenoviruses that have been genetically modified to produce replication-defective gene transfer vehicles. In these vehicles, the early adenovirus gene products ElA and ElB are deleted and provided *in trans* by the packaging cell line 293 developed by Frank Graham (Graham et al. (1987) *J. Gen. Birol.* 36:59-72 and Graham (1977) *J. Genetic Virology* 68:937-940). The gene to be transduced is commonly inserted into adenovirus in the deleted ElA and ElB

region of the virus genome Bett et al. (1994), *supra*. Adenovirus vectors as vehicles for efficient transduction of genes have been described by Stratford-Perricaudet (1990) *Human Gene Therapy* 1:2-256; Rosenfeld (1991) Science 252:431-434; Wang et al. (1991) *Adv. Exp. Med. Biol.* 309:61-66; Jaffe et al. (1992) *Nat Gent.* 1:372-378; Quantin et al. (1992) *Proc Natl. Acad. Sci. USA* 89:2581-2584; Rosenfeld et al. (1992) *Cell* 68:143-155; Stratford-Perricaudet et al. (1992) *J. Clin. Invest.* 90:626-630; Le Gal La Salle et al. (1993) *Science* 259:988-990; Mastrangeli et al. (1993) *J. Clin. Invest.* 91:225-234; Ragot et al. (1993) *Nature* 361:647-650; Hayaski et al. (1994) *J. Biol. Chem.* 269:23872-23875.

There are two major divisions of gene therapy protocols: *in vivo* and *ex vivo*. *In vivo* refers to administration of the therapeutic directly to the patient, usually by inhalation or injection, although oral administration has been suggested in some instances. *Ex vivo* gene therapy refers to the process of removing cells from a patient, for example in a biopsy, placing the cells into tissue culture, transferring genes to the cells in tissue culture, characterizing the newly genetically engineered cells, and finally returning the cells to the patient by intravenous infusion. Therapeutically, retroviruses are most often used for *ex vivo* transfer, whereas adenoviruses and liposomes are most often used for *in vivo* gene transfer.

In the treatment of cancer by replication-defective adenoviruses, the host immune response limits the duration of repeat doses of the therapeutic at two levels. First, the adenovirus delivery vehicle itself is immunogenic. Second, late virus genes are frequently expressed in transduced cells, eliciting cellular immunity. Thus, the ability to repeatedly administer cytokines, tumor suppressor genes, ribozymes or suicide genes is limited by the transient nature of gene expression, and the immunogenicity of both the gene transfer vehicle and the viral gene products of the transfer vehicle.

The first case, the immunogenicity of the vector, is akin to the problem facing mouse monoclonal antibodies complexed with bacterial toxins that are directed

against tumor-specific antigens. Use of these proteins as a therapeutic, popular a decade ago, failed due to the high doses required and ultimately, to immunogenicity. The same fate may befall replication-defective adenoviruses, unless the efficacy can be improved to achieve clinical useful therapeutic endpoints before immunogenicity limits repeat usage.

In the second case, steps have been taken to eliminate the unwanted transcription and expression of late adenovirus genes in transduced cells, with the resulting immunogenicity.

There is, therefore, substantial interest in being able to develop viral vectors which substantially reduce the present limitations and restrictions on the use of such vectors *in vivo*.

SUMMARY OF THE INVENTION

Replication-competent adenovirus vectors, and methods for their use as vehicles for the transduction of restricted cell types, are provided. The invention provides an adenovirus vector comprising an adenovirus gene under transcriptional control of a cell type-specific transcriptional regulatory element (TRE). In some embodiments, an adenoviral gene which is essential for replication is under transcriptional control of a cell type-specific transcriptional regulatory element (TRE). In one aspect, this adenoviral gene is an early gene. Additionally, one or more late genes and/or one or more transgenes may be under the control of a transcriptional initiation region that is transcriptionally active only in the target cells of interest. For these replication-competent adenovirus vectors, one or more of the promoters of the early and/or late genes essential for propagation is replaced with the transcriptional initiation region described above, where a transgene under a cell specific promoter may also be present.

The present invention further provides an adenovirus vector comprising a first adenovirus gene under transcriptional control of a cell type-specific TRE, and at least

a second gene under transcriptional control of a second cell type-specific TRE, wherein the first and the second cell type-specific TREs are substantially identical. In some embodiments, the second gene is an adenovirus gene. In preferred embodiments, the first adenovirus gene and the second gene are both adenovirus genes essential for replication. In these embodiments, the adenovirus vectors replicate preferentially in the target cell and, because the TREs controlling their expression are substantially identical, recombination can occur between the TREs, thus limiting the degree of propagation of the vector. In other embodiments, the second gene is a transgene.

The invention further provides host cells containing the adenovirus vectors of the invention.

The adenovirus vectors find use in the treatment of various indications and for making mammalian hosts that are transiently transgenic, and allowing for regulated adenovirus propagation and/or transgene expression, in parallel with the cellular regulation of the endogenous transcriptional initiation region. For the adenovirus which is transcriptionally competent in target cells, the adenovirus may be used to kill the cells, while optionally producing one or more proteins of interest. The vectors can also be useful for detecting the presence of cells that permit the function of a cell type-specific TRE in, for example, an appropriate biological (such as clinical) sample. Further, the adenovirus vector(s) can optionally selectively produce one or more proteins of interest in a target cell by using a cell type-specific TRE.

Accordingly, methods of using the adenoviral vectors of the invention are provided. In one aspect, methods are provided for using the adenovirus vectors described herein which entail introducing these vector(s) into a cell.

In another aspect, methods are provided for conferring selective cytoxicity on a cell which allows the cell type-specific TREs to function that entail contacting the cells with an adenovirus vector described herein, wherein the adenovirus vector enters the cell, and tumor growth is suppressed.

In another aspect, methods are provided for suppressing tumor growth, comprising contacting a target cell with an adenovirus vector described herein such that the adenovirus vector enters the cell.

In another aspect, methods are provided for modifying the genotype of a target cell, comprising contacting the cell with an adenovirus vector described herein, wherein the adenovirus vector enters the cell.

In yet another aspect, methods are provided for propagating the adenovirus vectors of the invention, comprising combining the adenovirus vectors with cells which allow the cell type-specific TREs to function, such that the adenovirus vector enters the cell and is propagated.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of various adenoviral vector constructs comprising AFP-TRE controlling expression of E1A, E1B or both, as described in Example 3.

Figures 2(A) and (B) are half tone reproductions depicting western analysis of E1A levels in CN733 (containing two AFP-TREs) and CN702 (control) infected cells. In Figure 2(A), the left panel shows Huh-7 (AFP+) cells; the right panel shows Dld-1 (AFP-) cells. In Figure 2(B), Sk-Hep-1 were the AFP- cells used.

Figures 3(A)-(C) are graphs depicting growth of CN733 in AFP producing (Huh-7; Figure 3(A)) and non-AFP producing (Sk-Hep-1, Figure 3(B); Dld-1, Figure 3(C)) cells.

Figures 4(A)-(C) are graphs depicting growth of CN732 (Fig. 4(A); solid diamonds), CN733 (Fig. 4(B); solid diamonds), and CN734 (Fig. 4(C); solid diamonds) in HepG2 cells, as compared to control CN702 (solid squares).

Figure 5(A)-(C) are graphs depicting growth of CN732 (Fig. 5(A); solid squares), and CN733 (Fig. 5(B); solid circles) in primary hepatocytes, compared to control CN702 (solid diamonds).

Figure 6(A)-(B) are graphs comparing tumor volume in mice harboring hepatocarcinoma cell line HepG2 and treated with CN733 (Fig. 5(A); squares) or with control buffer (circles). Fig. 6(A) depicts measuring tumor volume over a period of 43 days (six weeks). In Fig. 6(B), single intratumoral administration of CN733 ("B") was compared to five consecutive daily doses of CN733 ("J").

Figure 7 is a graph depicting serum AFP levels in tumor-bearing mice receiving CN733 (triangles) or receiving buffer (circles).

Figure 8 is a schematic representation of various adenoviral vector constructs comprising CEA-TRE controlling expression of E1A, E1B or both, as described in Example 4.

Figure 9 is a schematic representation of various adenoviral vector constructs comprising MUC-TRE controlling expression of E1A, E1B or both, as described in Example 5.

Figure 10 (SEQ ID NO:9) depicts depicts the sequence of the 5'-flanking region of the rat probasin (PB) gene, including the PB-TRE region. Numbers above the nucleotides indicate position relative to the transcription start site. The locations of androgen response elements (ARE) are indicated.

Figure 11 depicts schematic diagrams of various adenovirus vectors in which various genes are under control of a PB-TRE.

Figure 12 is a schematic representation of the *hKLK2*-TREs used to generate the adenoviral constructs described in Example 7.

Figure 13 is a schematic representation of the adenoviral constructs described in Example 7, in which adenoviral genes E1A and E1B are under transcriptional control of *hKLK2*-TREs. The ovals indicate that the endogenous E1A is present. The triangles indicate that the endogenous E1B promoter was removed. Abbreviations for TREs are as follows: hKLK2 P: *hKLK2* promoter; hKLK2 (1.8 E + P): 1.8 kb *hKLK2* enhancer and minimal *hKLK2* promoter, as depicted in Figure 12; hKLK2 (1.17 kb E

+ P): 1.17 kb *hKLK2* enhancer and minimal *hKLK2* promoter, as depicted in Figure 12.

Figure 14 depicts a nucleotide sequence of a carcinoembryonic antigen TRE.

Figure 15 depicts a nucleotide sequence of a prostate-specific antigen TRE.

Figure 16 depicts a nucleotide sequence of a human glandular kallikrein TRE.

Figure 17 depicts a nucleotide sequence of a mucin TRE.

Figure 18 depicts a nucleotide sequence of a rat probasin TRE.

Figure 19 depicts a nucleotide sequence and translated amino acid sequence of an adenovirus death protein.

MODES FOR CARRYING OUT THE INVENTION

We have discovered and constructed replication-competent adenovirus vectors containing cell type-specific transcriptional regulatory elements (TREs) which can preferentially replicate in cells that allow function of said TREs, and we have developed methods of using these adenovirus vectors. The adenovirus vectors of this invention comprise an adenovirus gene under the transcriptional control of a cell type-specific TRE. Preferably, the adenovirus gene is one that enhances, i.e. promotes, cell death, more preferably one that is essential for adenovirus replication. Preferably, the adenovirus gene necessary for cell replication is an early gene. In some embodiments, the adenovirus vectors of this invention comprise an adenovirus gene under the transcriptional control of a cell type-specific TRE, and at least one other gene, such as an adenoviral gene or a transgene, under control of a second cell type-specific TRE which is substantially identical to the first TRE. Preferably, the first and second genes under transcriptional control of the cell type-specific TREs are both adenovirus genes necessary for replication. By providing for cell-specific transcription through the use of one or more cell type-specific TREs, the invention provides adenovirus vectors that can be used for cell-specific cytotoxic effects due to selective replication.

The adenovirus vectors of the invention replicate preferentially in TRE functional cells (i.e., at a higher yield than in TRE non-functional cells), referred to herein as target cells. This replication preference is indicated by comparing the level of replication (i.e., titer) in cells in which the TRE is active to the level of replication in cells in which the TRE is not active (i.e., a non-target cell). The replication preference is even more significant, as the adenovirus vectors of the invention actually replicate at a significantly lower rate in TRE non-functional cells than wild type virus. Comparison of the adenovirus titer of a target cell to the titer of a TRE inactive cell type provides a key indication that the overall replication preference is enhanced due to the replication in target cells as well as depressed replication in non-target cells. This is especially useful in the cancer context, in which targeted cell killing is desirable.

Runaway infection is prevented due to the cell-specific requirements for viral replication and to this instability, which may be mediated by recombination between TREs. Without wishing to be bound by any particular theory, the inventors note that production of adenovirus proteins can serve to activate and/or stimulate the immune system, either generally or specifically toward target cells producing adenoviral proteins which can be an important consideration in the cancer context, where patients are often moderately to severely immunocompromised.

Adenovirus vectors that comprise at least two cell type-specific TREs which are substantially identical to one another, have been observed to be less stable than previously described adenovirus vectors which contain two TREs which are different from one another. This instability appears to be due to recombination between the TREs, and confers an advantage in that viral replication is limited. Adenoviral vector instability may be particulary desirable in certain contexts, such as when an automatic self-destruction property can shut down the virus, thereby controlling the degree of propagation. Without wishing to be bound by theory, such genome instability may be the result of homologous recombination through the duplicated TRE sequences.

Adenovirus vectors have been constructed in which each of the E1A and E1B genes have been placed under transcriptional control of the same cell type-specific TREs, for example, TREs from the *PSA* gene (*PSE*-TRE), the probasin gene (*PB*-TRE), the *hKLK2* gene (*hKLK2*-TRE), the a-fetoprotein gene (*AFP*-TRE), the carcinoembryonic antigen gene (*CEA*-TRE), and the mucin gene (*MUC*-TRE).

The adenovirus vectors, in which substantially identical cell type-specific TREs are used to control replication, achieve a high level of target cell specificity while displaying an instability which leads to limited propagation. Thus, an aspect of the invention uses and takes advantage of what has been considered an undesirable aspect of this type of adenoviral vectors, namely, their instability.

General Techniques

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, "Molecular Cloning: A Laboratory Manual", second edition (Sambrook et al., 1989); "Oligonucleotide Synthesis" (M.J. Gait, ed., 1984); "Animal Cell Culture" (R.I. Freshney, ed., 1987); "Methods in Enzymology" (Academic Press, Inc.); "Handbook of Experimental Immunology" (D.M. Weir & C.C. Blackwell, eds.); "Gene Transfer Vectors for Mammalian Cells" (J.M. Miller & M.P. Calos, eds., 1987); "Current Protocols in Molecular Biology" (F.M. Ausubel et al., eds., 1987); "PCR: The Polymerase Chain Reaction", (Mullis et al., eds., 1994); and "Current Protocols in Immunology" (J.E. Coligan et al., eds., 1991).

For techniques related to adenovirus, see, inter alia, Felgner and Ringold (1989) *Nature* 337:387–388; Berkner and Sharp (1983) *Nucl. Acids Res.* 11:6003–6020; Graham (1984) *EMBO J.* 3:2917–2922; Bett et al. (1993) *J. Virology* 67:5911–5921; Bett et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:8802–8806.

Definitions

An "adenovirus vector" or "adenoviral vector" (used interchangeably) is a term well understood in the art and generally comprises a polynucleotide (defined herein) comprising all or a portion of an adenovirus genome. As used herein, "adenovirus" refers to the virus itself or derivatives thereof. The term covers all serotypes and subtypes and both naturally occurring and recombinant forms, except where otherwise indicated. For the purposes of the present invention, an adenovirus vector contains a cell type-specific TRE operably linked to an adenovirus gene, and may optionally contain a second adenoviral gene or a transgene operably linked to a cell type-specific TRE or another type of TRE, which is non-cell type-specific. An adenoviral vector of the present invention can be in any of several forms, including, but not limited to, naked DNA; an adenoviral vector encapsulated in an adenovirus coat; packaged in another viral or viral-like form (such as herpes simplex virus and AAV); encapsulated in a liposome; complexed with polylysine or other biocompatible polymer; complexed with synthetic polycationic molecules; conjugated with transferrin; complexed with compounds such as PEG to immunologically "mask" the molecule and/or increase half-life, or conjugated to a non-viral protein. An adenoviral vector of this invention may be in the form of any of the delivery vehicles described herein. Such vectors are one embodiment of the invention. Preferably, the polynucleotide is DNA. As used herein, "DNA" includes not only bases A, T, C, and G, but also includes any of their analogs or modified forms of these bases, such as methylated nucleotides, internucleotide modifications such as uncharged linkages and thioates, use of sugar analogs, and modified and/or alternative backbone structures, such as polyamides. For purposes of this invention, adenovirus vectors are replication-competent in a target cell.

As used herein, a "transcriptional regulatory element", or "TRE" is a polynucleotide sequence, preferably a DNA sequence, that regulates (i.e., controls) transcription of an operably-linked polynucleotide sequence by an RNA polymerase to form RNA. As used herein, a TRE increases transcription of an operably linked

polynucleotide sequence in a host cell that allows the TRE to function. The TRE comprises an enhancer element and/or promoter element, which may or may not be derived from the same gene. The promoter and enhancer components of a TRE may be in any orientation and/or distance from the coding sequence of interest, and comprise multimers of the foregoing, as long as the desired transcriptional activity is obtained. As discussed herein, a TRE may or may not lack a silencer element.

An "enhancer" is a term well understood in the art and is a polynucleotide sequence derived from a gene which increases transcription of a gene which is operably-linked to a promoter to an extent which is greater than the transcription activation effected by the promoter itself when operably-linked to the gene, i.e. it increases transcription from the promoter. Having "enhancer activity" is a term well understood in the art and means what has been stated, i.e., it increases transcription of a gene which is operably linked to a promoter to an extent which is greater than the increase in transcription effected by the promoter itself when operably linked to the gene, i.e., it increases transcription from the promoter.

"Under transcriptional control" is a term well-understood in the art and indicates that transcription of a polynucleotide sequence, usually a DNA sequence, depends on its being operably (operatively) linked to an element which contributes to the regulation of, either promotes or inhibits, transcription.

The term "operably linked" relates to the orientation of polynucleotide elements in a functional relationship. A TRE is operably linked to a coding segment if the TRE promotes transcription of the coding sequence. Operably linked means that the DNA sequences being linked are generally contiguous and, where necessary to join two protein coding regions, contiguous and in the same reading frame. However, since enhancers generally function when separated from the promoter by several kilobases and intronic sequences may be of variable length, some polynucleotide elements may be operably linked but not contiguous.

A "cell type-specific TRE" is preferentially functional in a specific type of cell relative to other types of cells of different functionality. "Cell type" is a reflection of a differentiation state of a cell which is, under normal physiological conditions, an irreversible, end-stage state. For example, a prostate-specific antigen TRE is functional in prostate cells, but is not substantially functional in other cell types such as hepatocytes, astrocytes, cardiocytes, lymphocytes, etc. Generally, a cell type-specific TRE is active in only one cell type. When a cell type-specific TRE is active in more than one cell type, its activity is restricted to a limited number of cell types, i.e., it is not active in all cell types. A cell type-specific TRE may or may not be tumor cell specific.

As used herein, the term "cell type-specific" is intended to mean that the TRE sequences to which a gene, which may be a gene essential for replication of an adenoviral vector, is operably linked, or to which a transgene is operably linked, functions specifically in that target cell so that transcription (and replication, if the operably linked gene is one essential for adenovirus replication) proceeds in that target cell, or so that a transgene polynucleotide is expressed in that target cell. This can occur by virtue of the presence in that target cell, and not in non-target cells, of transcription factors that activate transcription driven by the operably linked transcriptional control sequences. It can also occur by virtue of the absence of transcription inhibiting factors that normally occur in non-target cells and prevent transcription driven by the operably linked transcriptional control sequences. The term "cell type-specific", as used herein, is intended to include cell type specificity, tissue specificity, as well as specificity for a cancerous state of a given target cell. In the latter case, specificity for a cancerous state of a normal cell is in comparison to a normal, non-cancerous counterpart.

As used herein, a TRE derived from a specific gene is referred to by the gene from which it was derived and is a polynucleotide sequence which regulates transcription of an operably linked polynucleotide sequence in a host cell that

expresses said gene. For example, as used herein, a "human glandular kallikrein transcriptional regulatory element", or "*hKLK2*-TRE" is a polynucleotide sequence, preferably a DNA sequence, which increases transcription of an operably linked polynucleotide sequence in a host cell that allows an *hKLK2*-TRE to function, such as a cell (preferably a mammalian cell, even more preferably a human cell) that expresses androgen receptor. An *hKLK2*-TRE is thus responsive to the binding of androgen receptor and comprises at least a portion of an *hKLK2* promoter and/or an *hKLK2* enhancer (i.e., the ARE or androgen receptor binding site).

As used herein, a "probasin (*PB*) transcriptional regulatory element", or "*PB*-TRE" is a polynucleotide sequence, preferably a DNA sequence, which selectively increases transcription of an operably-linked polynucleotide sequence in a host cell that allows a *PB*-TRE to function, such as a cell (preferably a mammalian cell, even more preferably a human cell) that expresses androgen receptor. A *PB*-TRE is thus responsive to the binding of androgen receptor and comprises at least a portion of a *PB* promoter and/or a *PB* enhancer (i.e., the ARE or androgen receptor binding site).

As used herein, a "prostate-specific antigen (*PSA*) transcriptional regulatory element", or "*PSA*-TRE", or "*PSE*-TRE" is polynucleotide sequence, preferably a DNA sequence, which selectively increases transcription of an operably linked polynucleotide sequence in a host cell that allows a *PSA*-TRE to function, such as a cell (preferably a mammalian cell, even more preferably a human cell) that expresses androgen receptor. A *PSE*-TRE is thus responsive to the binding of androgen receptor and comprises at least a portion of a *PSA* promoter and/or a *PSA* enhancer (i.e., the ARE or androgen receptor binding site).

As used herein, a "carcinoembryonic antigen (*CEA*) transcriptional regulatory element", or "*CEA*-TRE" is polynucleotide sequence, preferably a DNA sequence, which selectively increases transcription of an operably linked polynucleotide sequence in a host cell that allows a *CEA*-TRE to function, such as a cell (preferably a mammalian cell, even more preferably a human cell) that expresses CEA. The

CEA-TRE is responsive to transcription factors and/or co-factor(s) associated with CEA-producing cells and comprises at least a portion of the *CEA* promoter and/or enhancer.

As used herein, an "α-fetoprotein (*AFP*) transcriptional regulatory element", or "*AFP*-TRE" is polynucleotide sequence, preferably a DNA sequence, which selectively increases transcription (of an operably linked polynucleotide sequence) in a host cell that allows an *AFP*-TRE to function, such as a cell (preferably a mammalian cell, even more preferably a human cell) that expresses AFP. The *AFP*-TRE is responsive to transcription factors and/or co-factor(s) associated with AFP-producing cells and comprises at least a portion of the *AFP* promoter and/or enhancer.

As used herein, an "a mucin gene (*MUC*) transcriptional regulatory element", or "*MUC1*-TRE" is a polynucleotide sequence, preferably a DNA sequence, which selectively increases transcription (of an operably-linked polynucleotide sequence) in a host cell that allows an *MUC1*-TRE to function, such as a cell (preferably a mammalian cell, even more preferably a human cell) that expresses MUC1. The *MUC1*-TRE is responsive to transcription factors and/or co-factor(s) associated with MUC1-producing cells and comprises at least a portion of the *MUC1* promoter and/or enhancer.

As used herein, a "target cell" is one which allows (i.e., permits or induces) a cell type-specific TRE to function. Preferably, a target cell is a mammalian cell, preferably a human cell.

As used herein, "a cell which allows a TRE to function" or a cell in which the function of a TRE is "sufficiently preserved" or "functionally preserved", or "a cell in which a TRE is functional" is a cell in which the TRE, when operably linked to a promoter (if not included in the TRE) and a reporter gene, increases expression of the reporter gene at least about 2-fold, preferably at least about 5-fold, preferably at least about 10-fold, more preferably at least about 20-fold, more preferably at least about

50-fold, more preferably at least about 100-fold, more preferably at least about 200-fold, even more preferably at least about 400- to about 500-fold, even more preferably at least about 1000-fold, when compared to the expression of the same promoter and reporter gene when not operably linked to said TRE. Methods for measuring levels (whether relative or absolute) of expression are known in the art and are described herein.

The activity of a TRE generally depends upon the presence of transcriptional regulatory factors and/or the absence of transcriptional regulatory inhibitors. Transcriptional activation can be measured in a number of ways known in the art (and described in more detail below), but is generally measured by detection and/or quantitation of mRNA or the protein product of the coding sequence under control of (i.e., operatively linked to) the TRE. As discussed herein, a TRE can be of varying lengths, and of varying sequence composition. By transcriptional activation, it is intended that transcription will be increased above basal levels in the target cell by at least about 2-fold, preferably at least about 5-fold, preferably at least about 10-fold, more preferably at least about 20-fold. More preferably at least about 50-fold, more preferably at least about 100-fold, even more preferably at least about 200-fold, even more preferably at least about 400- to about 500-fold, even more preferably, at least about 1000-fold. Basal levels are generally the level of activity, if any, in a non-target cells, or the level of activity (if any) of a reporter construct lacking the TRE of interest as tested in a target cell type.

A "functionally-preserved" variant of a TRE is a TRE which differs from another TRE, but still retains ability to increase transcription of an operably linked polynucleotide, especially cell type-specific transcription activity. The difference in a TRE can be due to differences in linear sequence, arising from, for example, single or multiple base mutation(s), addition(s), deletion(s), and/or modification(s) of the bases. The difference can also arise from changes in the sugar(s), and/or linkage(s) between the bases of a TRE.

Certain point mutations within sequences of TREs have been shown to decrease transcription factor binding and gene activation. One of skill in the art would recognize that some alterations of bases in and around known the transcription factor binding sites are more likely to negatively affect gene activation and cell-specificity, while alterations in bases which are not involved in transcription factor binding are not as likely to have such effects. Certain mutations are also capable of increasing TRE activity. Testing of the effects of altering bases may be performed *in vitro* or *in vivo* by any method known in the art, such as mobility shift assays, or transfecting vectors containing these alterations in TRE functional and TRE non-functional cells. Additionally, one of skill in the art would recognize that point mutations and deletions can be made to a TRE sequence without altering the ability of the sequence to regulate transcription.

A "host cell" includes an individual cell or cell culture which can be or has been a recipient of any polynucleotide(s) and/or vector(s) of this invention. Host cells include progeny of a single host cell, and the progeny may not necessarily be completely identical (in morphology or of total DNA complement) to the original parent cell due to natural, accidental, or deliberate mutation and/or change. A host cell includes cells transfected or infected *in vivo* with a polynucleotide and/or a vector of this invention.

As used herein, the terms "neoplastic cells", "neoplasia", "tumor", "tumor cells", "cancer" and "cancer cells", (used interchangeably) refer to cells which exhibit relatively autonomous growth, so that they exhibit an aberrant growth phenotype characterized by a significant loss of control of cell proliferation. Neoplastic cells can be malignant or benign.

In the context of a viral vector, e.g., adenovirus vector(s), of the invention, a "heterologous" promoter or enhancer is one which is not present in wild-type virus. Examples of a heterologous promoter or enhancer are the albumin promoter or enhancer and other viral promoters and enhancers, such as SV40.

In the context of adenovirus vector(s), an "endogenous" promoter, enhancer, or TRE is native to or derived from adenovirus.

The term "gene" is well understood in the art and is a polynucleotide encoding a polypeptide. In addition to the polypeptide coding regions, a gene includes non-coding regions including, but not limited to, introns, transcribed but untranslated segments, and regulatory elements upstream and downstream of the coding segments.

In the context of adenovirus vector(s), a "heterologous polynucleotide" or "transgene" is any gene that is not present in wild-type adenovirus. Preferably, the transgene will also not be expressed or present in the target cell prior to introduction by the adenovirus vector. Examples of preferred transgenes are provided below.

A sequence, whether polynucleotide or polypeptide, "depicted in" a SEQ ID NO, means that the sequence is present as an identical contiguous sequence in the sequence of the SEQ ID NO.

The terms "polynucleotide" and "nucleic acid", used interchangeably herein, refer to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. These terms include a single-, double- or triple-stranded DNA, genomic DNA, cDNA, RNA, DNA-RNA hybrid, or a polymer comprising purine and pyrimidine bases, or other natural, chemically, biochemically modified, non-natural or derivatized nucleotide bases. The following are non-limiting examples of polynucleotides: a gene or gene fragment, exons, introns, mRNA, tRNA, rRNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs, uracyl, other sugars and linking groups such as fluororibose and thioate, and nucleotide branches. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling

component. Other types of modifications included in this definition are caps, substitution of one or more of the naturally occurring nucleotides with an analog, and introduction of means for attaching the polynucleotide to proteins, metal ions, labeling components, other polynucleotides, or a solid support. Preferably, the polynucleotide is DNA. As used herein, "DNA" includes not only bases A, T, C, and G, but also includes any of their analogs or modified forms of these bases, such as methylated nucleotides, internucleotide modifications such as uncharged linkages and thioates, use of sugar analogs, and modified and/or alternative backbone structures, such as polyamides.

A polynucleotide or polynucleotide region has a certain percentage (for example, 80%, 85%, 90%, or 95%) of "sequence identity" to another sequence means that, when aligned, that percentage of bases are the same in comparing the two sequences. This alignment and the percent homology or sequence identity can be determined using software programs known in the art, for example those described in *Current Protocols in Molecular Biology* (F.M. Ausubel et al., eds., 1987) Supplement 30, section 7.7.18, Table 7.7.1. A preferred alignment program is ALIGN Plus (Scientific and Educational Software, Pennsylvania), preferably using default parameters.

"Androgen receptor", or AR as used herein refers to a protein whose function is to specifically bind to androgen and, as a consequence of the specific binding, recognize and bind to an androgen response element (ARE), following which the AR is capable of regulating transcriptional activity. The AR is a nuclear receptor that, when activated, binds to cellular androgen-responsive element(s). In normal cells the AR is activated by androgen, but in non-normal cells (including malignant cells) the AR may be activated by non-androgenic agents, including hormones other than androgens. Encompassed in the term "androgen receptor" are mutant forms of an androgen receptor, as long as the function is sufficiently preserved. Mutants include androgen receptors with amino acid additions, insertions, truncations and deletions,

as long as the function is sufficiently preserved. In this context, a functional androgen receptor is one that binds both androgen and, upon androgen binding, an ARE.

"Replication" and "propagation" are used interchangeably and refer to the ability of an adenovirus vector of the invention to reproduce or proliferate. This term is well understood in the art. For purposes of this invention, replication involves production of adenovirus proteins and is generally directed to reproduction of adenovirus. Replication can be measured using assays standard in the art and described herein, such as a burst assay or plaque assay. "Replication" and "propagation" include any activity directly or indirectly involved in the process of virus manufacture, including, but not limited to, viral gene expression, production of viral proteins, nucleic acids or other components, packaging of viral components into complete viruses, and cell lysis.

A "gene essential for replication" is a gene whose transcription is required for the vector to replicate in a cell.

The terms "polypeptide", "peptide" and "protein" are used interchangeably to refer to polymers of amino acids of any length. These terms also include proteins that are post-translationally modified through reactions that include glycosylation, acetylation and phosphorylation.

As used herein, "cytotoxicity" is a term well understood in the art and refers to a state in which one or more of a cell's usual biochemical or biological functions are perturbed (i.e., inhibited or elevated). These activities include, but are not limited to, metabolism, cellular replication, DNA replication, transcription, translation, and uptake of molecules. "Cytotoxicity" includes cell death and/or cytolysis. Assays are known in the art which indicate cytotoxicity, such as dye exclusion, ^{3}H-thymidine uptake, and plaque assays. The term "selective cytotoxicity", as used herein, refers to the cytotoxicity conferred by an adenoviral vector of the present invention on a cell which allows a cell type-specific TRE to function when compared to the cytotoxicity

conferred by an adenoviral vector of the present invention on a cell which does not allow, or is less permissive for, the same TRE to function. Such cytoxicity may be measured, for example, by plaque assays, reduction or stabilization in size of a tumor comprising target cells, or the reduction or stabilization of serum levels of a marker characteristic of the tumor cells or a tissue-specific marker, e.g., a cancer marker such as prostate specific antigen.

As used herein, a "cytotoxic" gene is a gene whose expression in a cell, either alone or in conjunction with adenovirus replication, enhances the degree and/or rate of cytotoxic and/or cytolytic activity in the cell.

A "therapeutic" gene is a gene whose expression in a cell is associated with a desirable result. In the cancer context, this desirable result may be, for example, cytotoxicity, repression or slowing of cell growth, and/or cell death.

A "biological sample" encompasses a variety of sample types obtained from an individual and can be used in a diagnostic or monitoring assay. The definition encompasses blood and other liquid samples of biological origin, solid tissue samples such as a biopsy specimen or tissue cultures or cells derived therefrom and the progeny thereof. The definition also includes samples that have been manipulated in any way after their procurement, such as by treatment with reagents, solubilization, or enrichment for certain components, such as proteins or polynucleotides. The term "biological sample" encompasses a clinical sample, and also includes cells in culture, cell supernatants, cell lysates, serum, plasma, biological fluid, and tissue samples.

An "individual" is a vertebrate, preferably a mammal, more preferably a human. Mammals include, but are not limited to, rodents, primates, farm animals, sport animals, and pets.

An "effective amount" is an amount sufficient to effect beneficial or desired clinical results. An effective amount can be administered in one or more administrations. For purposes of this invention, an effective amount of an adenoviral

vector is an amount that is sufficient to palliate, ameliorate, stabilize, reverse, slow or delay the progression of the disease state.

As used herein, "treatment" is an approach for obtaining beneficial or desired clinical results. For purposes of this invention, beneficial or desired clinical results include, but are not limited to, alleviation of symptoms, diminishment of extent of disease, stabilized (i.e., not worsening) state of disease, preventing spread (i.e., metastasis) of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. "Treatment" can also mean prolonging survival as compared to expected survival if not receiving treatment.

"Palliating" a disease means that the extent and/or undesirable clinical manifestations of a disease state are lessened and/or time course of the progression is slowed or lengthened, as compared to not administering adenoviral vectors of the present invention.

Adenoviral vectors of the invention

Replication-competent adenovirus vehicles are provided. The viruses comprise at least one gene under the transcriptional control of a transcriptional initiation region (transcriptional regulatory, or response, element, TRE) specifically regulated by target host cells. The genes that are regulated by the specifically regulated transcriptional initiation region may be early or late adenovirus genes and/or transgenes. By providing for regulated transcription restricted to specific host cell targets, one can provide for adenoviruses that can be used as vehicles for introducing genetic capability into host target cells, as distinct from other host cell types. The transgenes serve to modify the genotype or phenotype of the target cell, in addition to any modification of the genotype or phenotype resulting from the presence of the adenovirus. With competent adenoviruses, proliferation of the adenovirus may be used for its cytotoxic effect.

There are a number of different types of adenovirus, such as Ad2, Ad5, and Ad40, which may differ to minor or significant degrees. Particularly, Ad5 and Ad40 differ as to their host cell tropism, as well as the nature of the disease induced by the virus. For the purpose of the subject invention, Ad5 will be exemplified.

The genes of the adenovirus that are of interest for the subject invention may be divided into two groups, the early genes and the late genes, the expression of the latter being controlled by the major late promoter. Of the early genes, there are ElA, ElB, E2, E3 and E4. The ElA gene is expressed immediately after viral infection (0-2h) and before any other viral genes. ElA protein acts as a trans-acting positive-acting transcriptional regulatory factor, and is required for the expression of the other early viral genes and the promoter proximal major late genes. Despite the nomenclature, the promoter proximal genes driven by the major late promoter are expressed during early times after Ad5 infection. In the absence of a functional ElA gene, viral infection does not proceed, because the gene products necessary for viral DNA replication are not produced.

The ElB protein functions *in trans* and is necessary for transport of late mRNA from the nucleus to the cytoplasm. Defects in ElB expression result in poor expression of late viral proteins and an inability to shut off host cell protein synthesis.

The E4 gene has a number of transcription products. Open reading frames (ORF) 3 and ORF 6 of the E4 transcription unit increase the accumulation of major late transcription unit mRNAs by binding the 55-kDa protein from ElB and heterodimers of E2F-1 and DP-1. In the absence of functional protein from ORF3 and ORF6, plaques are produced with an efficiency less than 10^{-6} of that of wild type virus.

The major late genes relevant to the subject invention are genes such as Ll, L2 and L3, which encode proteins of the AD5 virus virion.

Regions of the adenovirus which may be deleted, usually at least 500nt, more usually at least about lknt, include in the AD5 genome nucleotides 300 to 3600 in El,

particularly 342 to 3523; 27000 to 31000, particularly 28133 to 30818 or 27865 to 30995 in E3. The deletion will be at least sufficient for insertion of the desired construct and allow for packaging.

The subject vectors can be used for a wide variety of purposes. The purpose will vary with the target cell. Suitable target cells are characterized by the transcriptional activation of the cell specific transcriptional response element in the adenovirus vehicle. The transcription initiation region will usually be activated in less than about 5%, more usually less than about 1%, and desirably by less than about 0.1% of the cells in the host.

Regulation of transcriptional activation is the result of interaction between transcriptional activators bound to cis-regulatory elements, factors bound to basal transcriptional elements and the activity of transcriptional mediators, or coactivators. The absence or presence of any of these factors may affect the level of transcription. Additionally, factors may be present in an inactive form, where the factors are activated through chemical modification, particularly as the result of a cellular signaling mechanism. In some cases, signaling molecules are able to act directly to activate transcription. Any of these mechanisms may operate to limit the types of cells in which the vehicle transcription initiation region is active.

It will be understood by one of skill in the art that very low basal levels of transcription may be present in non-targeted cell types. By transcriptional activation, it is intended that transcription will be increased above basal levels in the target cell by at least about 100 fold, more usually by at least about 1000 fold.

The cell specific response element, also referred to herein as a cell type-specific transcriptional response element (TRE), may be used with an adenovirus gene that is essential for propagation, so that replication competence is only achievable in the target cell, and/or with a transgene for changing the phenotype of the target cell. By transgene it is intended any gene that is not present in wild-type

adenovirus, frequently the transgene will also not be expressed in the target cell, prior to introduction by the adenovirus.

As exemplified by employing a cell specific response element comprising a promoter and enhancer construct specific for prostate cells, various genetic capabilities may be introduced into prostate cells expressing prostate specific antigen. Of particular interest is the opportunity to introduce cytotoxic effects that are controlled by a transcriptional initiation region specifically active in prostate cells. Other cell types that have specific active transcription factors associated with a state for which modulation is desirable include leukocytes, particularly lymphocytes, epithelial cells, endothelial cells, hepatic cells, pancreatic cells, neuronal cells, and keratinocytes. Since the adenovirus results in transient expression (approximately 6 to 8 weeks), one can provide transient capability to cells, where the desired result only requires a limited period for response.

Accordingly, the invention provides an adenovirus vector comprising an adenovirus gene under transcriptional control of a cell type-specific TRE. In some embodiments, a first cell type-specific transcriptional response element controls expression of a first adenovirus gene, and a second cell type-specific transcriptional response element controls expression of a seond gene, wherein the first transcriptional response element and the second transcriptional response element are substantially identical. The genes to be controlled under these TREs are preferably adenoviral genes essential for propagation, more preferably early genes. Alternatively, the genes to be controlled under these TREs may be a gene essential for propagation and a transgene. In these embodiments, the TREs are substantially identical. By "substantially identical" is meant a requisite degree of sequence identity between the two TREs. The degree of sequence identity between these TREs is at least about 75%, preferably at least about 80%, preferably at least about 85%, more preferably at least about 90%, even more preferably at least about 95%, even more preferably at least about 98%, and most preferably 100%. Sequence identity

can be determined by a sequence comparison using, i.e., sequence alignment programs that are known in the art, such as those decsribed in *Current Protocols in Molecular Biology* (F.M. Ausubel et al., eds., 1987) Supplement 30, section 7.7.18, Table 7.7.1 A preferred alignment program is ALIGN Plus (Scientific and Educational Software, Pennsylvania), preferably using default parameters. Alternatively, hybridization under stringent conditions can also indicate degree of sequence identity. As another way of determining requisite sequence identity, a construct containing these TREs can be tested for recombinant loop-out events by using techniques well known in the art such as southern hybridization. We have observed that constructs using these substantially identical TREs, particularly in conjunction with adenovirus early genes, display an instability which may be desirable in certain contexts, such as when an automatic "self-destruction" property can shut down the virus, thereby controlling the degree of propagation.

Accordingly, the invention includes an adenovirus vector comprisng a first adenovirus gene under transcriptional control of a first transcriptional regulatory element (TRE) and at least a second gene under transcriptional control of a second TRE, wherein the first TRE and the second TRE are substantially identical, and wherein the first TRE and second TRE are cell (i.e., cell or tissue) specific. It is understood that there may or may not be additional TREs in these adenoviral vectors, and that these additional TREs may or may not be subtantially identical to the first and/or second TREs. Accordingly, the invention includes use of three or more, four or more, TREs.

A cell type-specific TRE can also comprise multimers. For example, a cell type-specific TRE can comprise a tandem series of at least two, at least three, at least four, or at least five TREs. These multimers may also contain heterologous promoter and/or enhancer sequences.

A cell type-specific TRE may or may not lack a silencer. The presence of a silencer (i.e., a negative regulatory element) may assist in shutting off transcription

(and thus replication) in non-permissive cells (i.e., cell in a normal cell state). Thus, presence of a silencer may confer enhanced cell status-specific replication by more effectively preventing adenoviral vector replication in non-target cells. Alternatively, lack of a silencer may assist in effecting replication in target cells, thus conferring enhanced cell type-specific replication due to more effective replication in target cells.

In one embodiment, two substantially identical TREs control transcription of adenovirus early genes, preferably E1A and E1B. It is understood, however, that any of a number of combinations of genes may be used with these at least two TREs. Other preferred embodiments include those which contain substantially identical TREs that drive expression of E1A, E1B, and E4. Such constructs may or may not additionally contain a suitable or desired transgene, which may or may not be under control of a substantially identical TRE. Preparation of these and other embodiments employing substantially identical TREs are provided below and in the examples.

In embodiments in which two cell type-specific TREs are used, the invention does not require that the TREs be derived from the same gene. As long as the TRE sequences are substantially identical, and the requisite functionality is displayed, the TREs may be derived from different genes.

Any of the cell type-specific TREs may be used, as long as they are substantially identical with respect to each other. Accordingly, the TREs used can include, but are not limited to: (a) prostate specific TREs, such as derived from PSA or rat probasin; (b) liver specific TREs, such as alpha-feto protein; (c) carcinoembryonic antigen (CEA); (d) mucin; (e) any other cell-type specific TRE. A number of these TREs are known in the art and need not be described herein. Examples of cell type-specific TREs are provided below.

Cell type-specific transcriptional response elements

Depending upon the target cell type, various enhancers may be used to provide for target cell specific transcription. With lymphocytes, for B cells one may

use the Ig enhancer, for T cells one may use the T cell antigen receptor promoter. For the different muscle cells, one may use the promoters for the different myosins. For endothelial cells, one may use the different promoters for the different selectins. For each type of cell, there will be specific proteins associated with the cell, which allows for target cell specific transcription.

In one embodiment, the invention includes adenovirus vectors wherein the substantially identical TREs are prostate cell specific. For example, TREs that function preferentially in prostate cells and can be used in the present invention to target adenovirus replication to prostate neoplasia, include, but are not limited to, TREs derived from the prostate-specific antigen gene (*PSA*-TRE), the glandular kallikrein-1 gene (from the human gene, *hKLK2*-TRE), and the probasin gene (*PB*-TRE). All three of these genes are preferentially expressed in prostate cells and the expression is androgen-inducible. Generally, expression of genes responsive to androgen induction requires the presence of an androgen receptor (AR).

The region of the *PSA* gene that is used to provide cell specificity dependent upon androgens, particular in prostate cells, involves approximately 6.0 kilobases. Schuur et al. (1996) *J. Biol. Chem.* 271:7043-7051. An enhancer region of approximately 1.5 kb in humans is located between nt -5322 and nt -3739, relative to the transcription start site of the *PSA* gene. The *PSA* promoter consists of the sequence from about nt -540 to nt +8 relative to the transcription start site. Juxtapositioning of these two genetic elements yields a fully functional, minimal prostate-specific enhancer/promoter (*PSE*) TRE. Other portions of the approximately 6.0 kb region of the *PSA* gene can be used in the present invention, as long as requisite functionality is maintained.

The *PSE* and *PSA* TRE depicted in (SEQ ID NO:1) is the same as that given in GenBank Accession No. U37672, and published. Schuur et al. (1996). A variant *PSA*-TRE nucleotide sequence is depicted in (SEQ ID NO:2). This is the *PSA*-TRE contained within CN706 clone 35.190.13. CN706 is an adenoviral vector in which

the E1A gene in Ad5 is under transcriptional control of a *PSA*-TRE. CN706 demonstrates selective cytotoxicity toward PSA-expressing cells *in vitro* and *in vivo*. Rodriguez et al. (1997). CN706 was passaged through 293 and LNCaP cells. A clone, designated 35.190.13 was isolated. The structure of this clone was confirmed by PCR, restriction endonuclease digestion and Southern blotting. Both DNA strands of the CN706 clone 35.190.13 were sequenced between positions 1 and 3537. Seven single base pair changes were found in the PSE, compared to the sequence reported by Schuur et al. (1996). These point mutations are not in the ARE and are thus not likely to affect the function of the enhancer. One mutation was found in the *PSA* promoter region, but is not likely to affect gene expression from this promoter. In addition to these mutations, a missense mutation was found in the first exon of E1A. This C to G transition at position 3032 results in a Glu to Arg change in the E1A protein sequence. This mutation does not appear to diminish E1A function.

The region that is employed to provide cell specificity dependent upon androgens, particularly in prostate cells, involves an approximately 1.5kb enhancer region and a 0.5kb promoter region. The enhancer region in humans is located between nt -5322 and nt -3739, relative to the transcription start site of the prostate specific antigen (PSA) gene. The promoter consists of nt -540 to nt +8. Juxtaposition of the two genetic elements yields a fully functional, minimal prostate-specific enhancer promoter (PSE). The enhancer contains three regions that bind prostate-specific DNA binding proteins, one of which contains a putative androgen response element. The promoter region contains typical TATA and CAAT boxes as well as a second putative androgen response element.

Human glandular kallikrein (*hKLK2*, encoding the hK2 protein) is expressed exclusively in the prostate and its expression is up-regulated by androgens primarily by transcriptional activation. Wolf et al. (1992) *Molec. Endocrinol.* 6:753-762. Morris (1989) *Clin. Exp. Pharm. Physiol.* 16:345-351; Qui et al. (1990) *J. Urol.* 144:1550-1556; Young et al. (1992) *Biochem.* 31:818-824. The levels of hK2 found

in various tumors and in the serum of patients with prostate cancer differ substantially from those of PSA and indicate that hK2 antigen may be a significant marker for prostate cancer. Circulating hK2 in different relative proportions to PSA has been detected in the serum of patients with prostate cancer. Charlesworth et al. (1997) *Urology* 49:487-493. Expression of hK2 has been detected in each of 257 radical prostatectomy specimens analyzed. Darson et al. (1997) *Urology* 49:857-862. The intensity and extent of hK2 expression, detected using specific antibodies, increased from benign epithelium to high-grade prostatic intraepithelial neoplasia (PIN) and adenocarcinoma, whereas PSA and prostate acid phosphatase displayed an inverse pattern of immunoreactivity. Darson et al. (1997). Indeed, it has been reported that a certain percentage of PSA-negative tumors have detectable hK2. Tremblay et al. (1997) *Am. J. Pathol.* 150:455-459.

The activity of the *hKLK2* 5' promoter has been previously described and a region up to -2256 relative to the transcription start site was previously disclosed. Schedlich et al. (1987) *DNA* 6:429-437. The *hKLK2* promoter is androgen responsive and, in plasmid constructs wherein the promoter alone controls the expression of a reporter gene, expression of the reporter gene is increased approximately 10-fold in the presence of androgen. Murtha et al. (1993) *Biochem.* 32:6459-6464. *hKLK2* enhancer activity is found within a polynucleotide sequence approximately nt -12,014 to nt -2257 relative to the start of transcription (depicted in SEQ ID NO:3) and, when this sequence is operably linked to an *hKLK2* promoter and a reporter gene, transcription of operably-linked sequences in prostate cells increases in the presence of androgen at levels approximately 30- to approximately 100-fold over the level of transcription in the absence of androgen. This induction is generally orientation independent and position independent. Enhancer activity has also been demonstrated in the following regions (all relative to the transcription start site): about nt -3993 to about nt -3643 (nt 8021 to 8371 of SEQ ID NO:3), about nt -4814 to about nt -3643 (nt 7200 to 8371 of SEQ ID NO:3), about nt -5155 to about nt -3387 (nt 6859 to 8627

of SEQ ID NO:3), about nt -6038 to about nt -2394 (nt 5976 to 9620 of SEQ ID NO:3).

Thus, an *hKLK2* enhancer can be operably linked to an *hKLK2* promoter or a heterologous promoter to form an *hKLK2* transcriptional regulatory element (*hKLK2*-TRE). An *hKLK2*-TRE can then be operably linked to a heterologous polynucleotide to confer *hKLK2*-TRE-specific transcriptional regulation on the linked gene, thus increasing its expression.

The rat probasin (*PB*) gene encodes a nuclear and secreted protein, probasin, that is only expressed in the dorsolateral prostate. Dodd et al. (1983) *J. Biol. Chem.* 258:10731–10737; Matusik et al. (1986) *Biochem. Cell. Biol.* 64: 601-607; and Sweetland et al. (1988) *Mol. Cell. Biochem.* 84: 3-15. The dorsolateral lobes of the murine prostate are considered the most homologous to the peripheral zone of the human prostate, where approximately 68% of human prostate cancers are thought to originate.

A *PB*-TRE has been shown in an approximately 0.5 kb fragment of sequence upstream of the probasin coding sequence, from about nt -426 to about nt +28 relative to the transcription start site,as depicted in (SEQ ID NO:4). This minimal promoter sequence from the PB gene appears to provide sufficient information to direct development and hormone -regulated expression of an operably linked heterologous gene specifically to the prostate in transgenic mice. Greenberg et al. (1994) *Mol. Endocrinol.* 8:230-239.

In the present invention, replication-competent adenovirus vectors directed at specific target cells may also be generated with the use of TREs that are preferentially functional in the target tumor cells. Non-limiting examples of tumor cell-specific TREs, and non-limiting examples of respective potential target cells, include TREs from the following genes: α-fetoprotein (*AFP*) (liver cancer); mucin-like glycoprotein DF3 (*MUC1*) (breast carcinoma); carcinoembryonic antigen (*CEA*) (colorectal, gastric, pancreatic, breast, and lung cancers); plasminogen activator.

urokinase (*uPA*) and its receptor gene (breast, colon, and liver cancers); and *HER-2/neu* (*c-erbB2/neu*) (breast, ovarian, stomach, and lung cancers).

AFP is an oncofetal protein, the expression of which is primarily restricted to developing tissues of endodermal origin (yolk sac, fetal liver, and gut), although the level of its expression varies greatly depending on the tissue and the developmental stage. AFP is of clinical interest because the serum concentration of AFP is elevated in a majority of hepatoma patients, with high levels of AFP found in patients with advanced disease. The serum AFP levels in patients appear to be regulated by AFP expression in hepatocellular carcinoma but not in surrounding normal liver. Thus, the AFP gene appears to be regulated to hepatoma cell-specific expression.

Cell-specific TREs from the *AFP* gene have been identified. For example, the cloning and characterization of human AFP-specific enhancer activity is described in Watanabe et al. (1987) *J. Biol. Chem.* 262:4812-4818. The entire 5' *AFP* flanking region (containing the promoter, putative silencer, and enhancer elements) is contained within approximately 5 kb upstream from the transcription start site (SEQ ID NO:5).

The *AFP* enhancer region in human is located between about nt -3954 and about nt -3335, relative to the transcription start site of the *AFP* gene. The human *AFP* promoter encompasses a region from about nt -174 to about nt +29. Juxtapositioning of these two genetic elements, as depicted in SEQ ID NO:6, yields a fully functional *AFP*-TRE. Ido et al. (1995) describe a 259 bp promoter fragment (nt -230 to nt +29) that is specific for HCC. *Cancer Res.* 55:3105-3109. The *AFP* enhancer contains two regions, denoted A and B, located between nt -3954 and nt -3335 relative to the transcription start site. The promoter region contains typical TATA and CAAT boxes. Preferably, the *AFP*-TRE contains at least one enhancer region. More preferably, the *AFP*-TRE contains both enhancer regions.

Suitable target cells for adenoviral vectors containing *AFP*-TREs are any cell type that allow an *AFP*-TRE to function. Preferred are cells that express, or produce,

AFP, including, but not limited to, tumor cells expressing AFP. Examples of such cells are hepatocellular carcinoma cells, gonadal and other germ cell tumors (especially endodermal sinus tumors), brain tumor cells, ovarian tumor cells, acinar cell carcinoma of the pancreas (Kawamoto et al. (1992) *Hepatogastroenterology* 39:282–286), primary gall bladder tumor (Katsuragi et al. (1989) *Rinsko Hoshasen* 34:371–374), uterine endometrial adenocarcinoma cells (Koyama et al. (1996) *Jpn. J. Cancer Res.* 87:612-617), and any metastases of the foregoing (which can occur in lung, adrenal gland, bone marrow, and/or spleen). In some cases, metastatic disease to the liver from certain pancreatic and stomach cancers produce AFP. Especially preferred are hepatocellular carcinoma cells and any of their metastases. AFP production can be measured using assays standard in the art, such as RIA, ELISA or Western blots (immunoassays) to determine levels of AFP protein production or Northern blots to determine levels of AFP mRNA production. Alternatively, such cells can be identified and/or characterized by their ability to activate transcriptionally an *AFP*-TRE (i.e., allow an *AFP*-TRE to function).

The protein urokinase plasminogen activator (uPA) and its cell surface receptor, urokinase plasminogen activator receptor (uPAR), are expressed in many of the most frequently occurring neoplasia and appear to represent important proteins in cancer metastasis. Both proteins are implicated in breast, colon, prostate, liver, renal, lung and ovarian cancer. Transcriptional regulatory elements that regulate uPA and uPAR transcription have been extensively studied. Riccio et al. (1985) *Nucleic Acids Res.* 13:2759-2771; Cannio et al., (1991) *Nucleic Acids Res.* 19:2303–2308.

CEA is a 180,000-Dalton glycoprotein tumor-associated antigen present on endodermally-derived neoplasia of the gastrointestinal tract, such as colorectal, gastric (stomach) and pancreatic cancer, as well as other adenocarcinomas such as breast and lung cancers. CEA is of clinical interest because circulating CEA can be detected in the great majority of patients with CEA-positive tumors. In lung cancer, about 50% of total cases have circulating CEA, with high concentrations of CEA

(greater than 20 ng/ml) often detected in adenocarcinomas. Approximately 50% of patients with gastric carcinoma are serologically positive for CEA.

The 5' upstream flanking sequence of the *CEA* gene has been shown to confer cell-specific activity. The *CEA* promoter region, approximately the first 424 nucleotides upstream of the translational start site in the 5' flanking region of the gene, was shown to confer cell-specific activity when the region provided higher promoter activity in CEA-producing cells than in non-producing HeLa cells.. Schrewe et al. (1990) *Mol. Cell. Biol.* 10:2738-2748. In addition, cell-specific enhancer regions have been found. WO/95/14100. The entire 5' *CEA* flanking region (containing the promoter, putative silencer, and enhancer elements) appears to be contained within approximately 14.5 kb upstream from the transcription start site. Richards et al. (1995); WO 95/14100. Further characterization of the 5' flanking region of the *CEA* gene by Richards et al. (1995) indicated two upstream regions, -13.6 to -10.7 kb or -6.1 to -4.0 kb, when linked to the multimerized promoter resulted in high-level and selective expression of a reporter construct in CEA-producing LoVo and SW1463 cells. Richards et al. (1995) also localized the promoter region to nt -90 and nt +69 relative to the transcriptional start site, with region nt -41 to nt -18 as essential for expression. WO95/14100 describes a series of 5' flanking *CEA* fragments which confer cell-specific activity, such as about nt -299 to about nt +69; about nt -90 to about nt +69; nt -14,500 to nt -10,600; nt -13,600 to nt -10,600, nt -6100 to nt -3800. In addition, cell specific transcription activity is conferred on an operably linked gene by the *CEA* fragment from nt -402 to nt +69, depicted in (SEQ ID NO:7). Any *CEA*-TREs used in the present invention are derived from mammalian cells, including but not limited to, human cells. Thus, any of the *CEA*-TREs may be used in the invention as long as requisite desired functionality is displayed in the adenovirus vector. The cloning and characterization of *CEA* sequences have been described in the literature and are thus made available for practice of this invention and need not be described in detail herein.

The protein product of the *MUC1* gene (known as mucin or MUC1 protein; episialin; polymorphic epithelial mucin or PEM; EMA; DF3 antigen; NPGP; PAS-O; or CA15.3 antigen) is normally expressed mainly at the apical surface of epithelial cells lining the glands or ducts of the stomach, pancreas, lungs, trachea, kidney, uterus, salivary glands, and mammary glands. Zotter et al. (1988) *Cancer Rev.* 11–12: 55–101; and Girling et al. (1989) *Int. J. Cancer* 43: 1072–1076. However, mucin is overexpressed in 75–90% of human breast carcinomas. Kufe et al. (1984) *Hybridoma* 3: 223–232. For reviews, see Hilkens (1988) *Cancer Rev.* 11–12: 25–54; and Taylor-Papadimitriou, et al. (1990) *J. Nucl. Med. Allied Sci.* 34: 144–150. Mucin protein expression correlates with the degree of breast tumor differentiation. Lundy et al. (1985) *Breast Cancer Res. Treat.* 5: 269–276. This overexpression appears to be controlled at the transcriptional level.

Overexpression of the *MUC1* gene in human breast carcinoma cells MCF-7 and ZR-75-1 appears to be regulated at the transcriptional level. Kufe et al. (1984); Kovarik (1993) *J. Biol. Chem.* 268:9917–9926; and Abe et al. (1990) *J. Cell. Physiol.* 143: 226–231. The regulatory sequences of the *MUC1* gene have been cloned, including the approximately 0.9 kb upstream of the transcription start site which contains a TRE that appears to be involved in cell-specific transcription, depicted in SEQ ID NO:8. Abe et al. (1993) *Proc. Natl. Acad. Sci. USA* 90: 282-286; Kovarik et al. (1993); and Kovarik et al. (1996) *J. Biol. Chem.* 271:18140–18147.

Any *MUC1*-TREs used in the present invention are derived from mammalian cells, including but not limited to, human cells. Preferably, the *MUC1*-TRE is human. In one embodiment, the *MUC1*-TRE may contain the entire 0.9 kb 5' flanking sequence of the *MUC1* gene. In other embodiments, the *MUC1*-TREs comprise the following sequences (relative to the transcription start site of the *MUC1* gene): about nt -725 to about nt +31, nt -743 to about nt +33, nt -750 to about nt +33, and nt -598 to about nt +485 (operably-linked to a promoter).

The *c-erbB2/neu* gene (*HER-2/neu* or *HER*) is a transforming gene that encodes a 185 kD epidermal growth factor receptor-related transmembrane glycoprotein. In humans, the c-erbB2/neu protein is expressed during fetal development, however, in adults, the protein is weakly detectable (by immunohistochemistry) in the epithelium of many normal tissues. Amplification and/or over-expression of the *c-erbB2/neu* gene has been associated with many human cancers, including breast, ovarian, uterine, prostate, stomach and lung cancers. The clinical consequences of the c-erbB2/neu protein over-expression have been best studied in breast and ovarian cancer. c-erbB2/neu protein over-expression occurs in 20 to 40% of intraductal carcinomas of the breast and 30% of ovarian cancers, and is associated with a poor prognosis in subcategories of both diseases. Human, rat and mouse *c-erbB2/neu* TREs have been identified and shown to confer *c-erbB2/neu* expressing cell specific activity. Tal et al. (1987) *Mol. Cell. Biol.* 7:2597–2601; Hudson et al. (1990) *J. Biol. Chem.* 265:4389–4393; Grooteclaes et al. (1994) *Cancer Res.* 54:4193–4199; Ishii et al. (1987) *Proc. Natl. Acad. Sci. USA* 84:4374–4378; Scott et al. (1994) *J. Biol. Chem.* 269:19848–19858.

In the present invention, cell type-specific TREs which are tumor-specific may be used in conjunction with other, non-tumor-specific cell type-specific TREs from the following exemplary genes (tissue in which the TREs are specifically functional are in parentheses): vascular endothelial growth factor receptor (endothelium); albumin (liver); factor VII (liver); fatty acid synthase (liver); von Willebrand factor and flt-1 (endothelium); alpha-actin and myosin heavy chain (both in smooth muscle); synthetase I (small intestine); Na-K-Cl transporter (kidney). Additional cell type-specific TREs are known in the art.

Additional tumor- and/or cell type-specific TREs known in the art include the following: aromatase, mammary gland-specific promoter, mammaglobin, urokinase, and human alpha-lactalbumin (breast tissue); BCSG1, BRCA1, and BRCA2 (breast cancer); human papilloma virus (HPV) cell type dependent regulatory element

(cervical cancer); BLCA4 (bladder cancer); uroplakin (bladder); NCA (gastric cancer); hypoxanthine phosphoribosyltransferase (HPRT) (glioma); AVP, human pulmonary surfactant protein B gene, and puromycin N-acetyltransferase (lung cancer); tyrosinase, gp100, tyrosinase related proteins 1 and 2; MART-1, and melanocyte specific factory (MSF) (melanoma); HER2/neu, urokinase, and CA125 (ovarian cancer); SL3-3 and T cell antigen receptor (T cell lymphoma); and prostatic acid phosphatase (prostate). Descriptions of these cell-specific TREs can be found in various publications, including the following: Zhou et al. (1996) J. Biol. Chem. 271:15164-15202 (aromatase); International Patent Application No. WO 98/15634 (mammary gland-specific promoter); Watson et al. (1996) *Cancer Res.* 56:860-865 (mammaglobin); Ji et al. (1997) *Cancer Res.* 57:759-764 (breast cancer-specific gene BCSG1); (1995) *Gene* 159:65-71 (HER-2/neu); Cannio et al. (1991) *Nucl. Acids Res.* 19:2303-2308 (urokinase); (1993) *Virol.* 195:500-510 (HPV cell type dependent regulatory element); Rincon-Limas et al. (1994) J. Neurosci. Res. 38:259-267 (HPRT); (1992) *Gene* 117:255-258 (puromycin N-acetyltransferase); Bohinski et al. (1993) *J. Biol. Chem.* 268:11160-11166 (human pulmonary surfactant protein B gene); Vile et al. (1993) *Cancer Res.* 53:3860-3864 (tyrosinase); Butterfield et al. (1997) *Gene* 191:129-134 (MART-1); Yavuzer et al. (1994) *Mol. Cell. Biol.* 14:3494-3503 (MSF); Garcia-Arenas et al. (1995) *Mol. Cell. Endocrinol.* 111:29-37 (prostatic acid phosphatase); Boral et al. (1989) *J. Virol.* 63:76-84 (SL3-3); and (1990) *Science* 247:1225-1229.

The TREs listed above are provided as non-limiting examples of TREs that would function in the instant invention. Additional cell-specific TREs are known in the art, as are methods to identify and test cell specificity of suspected TREs.

Activity of a TRE can be determined as follows. A TRE polynucleotide sequence or set of such sequences can be generated using methods known in the art, such as chemical synthesis, site-directed mutagenesis, PCR, and/or recombinant methods. The sequence(s) to be tested can be inserted into a vector containing a

promoter (if no promoter element is present in the TRE) and an appropriate reporter gene encoding a reporter protein, including, but not limited to, chloramphenicol acetyl transferase (CAT), β-galactosidase (encoded by the *lacZ* gene), luciferase (encoded by the *luc* gene), alkaline phosphatase, green fluorescent protein, and horse radish peroxidase. Such vectors and assays are readily available, from, inter alia, commercial sources. Plasmids thus constructed are transfected into a suitable host cell to test for expression of the reporter gene as controlled by the putative TRE using transfection methods known in the art, such as calcium phosphate precipitation, electroporation, liposomes (lipofection), and DEAE dextran.

After introduction of the TRE-reporter gene construct into a host cell under appropriate conditions, TRE activity may be measured by detection and/or quantitation of reporter gene-derived mRNA or protein product. The reporter gene protein can be detected directly (e.g., immunochemically) or through its enzymatic activity, if any, with an appropriate substrate. Generally, to determine cell specific activity of a TRE, the TRE-reporter gene constructs are introduced into a variety of cell types. The amount of TRE activity is determined in each cell type and compared to that of a reporter gene construct without the TRE. A TRE is cell specific when it is preferentially functional in a specific type of cell over a different type of cell.

For example, the specificity of *PB*-TRE activity for prostate cell that express the androgen receptor (AR) was demonstrated as follows. The region of the PB 5'-flanking DNA (nt -426 to nt +28) (SEQ ID NO:9) including the endogenous promoter sequences was inserted upstream of the firefly luciferase gene to generate a chimeric *PB*-TRE-luc plasmid. Cationic-mediated, transient transfection of LNCaP (PSA-producing and AR-producing prostate carcinoma cells) and PC-3 (PSA-deficient and AR-deficient prostate carcinoma cells) cells was performed. The results showed that LNCaP cells transfected with *PB*-TRE-luc had approximately 400 times more activity than untransfected cells, indicating that the *PB*-TRE was intact. Further, the overall luciferase activity recovered in the cellular extracts of transfected LNCaP cells was

about 30-40-fold higher than that measured in the cellular extracts of transfected PC-3 cells. Thus, the results indicate that *PB*-TRE expression is preferentially functional in PSA-producing, AR-producing prostate carcinoma cells as compared to PSA-deficient, AR-deficient prostate carcinoma cells and that *PB*-TRE is capable of mediating specific expression in cells producing the androgen receptor.

Transgenes

Use of competent adenovirus, which is competent in particular target cells, allow for proliferation of the adenovirus in the target cells resulting in the death of the host cells and proliferation of the adenovirus to other host cells. To further ensure cytotoxicity, one may have one or more transgenes present which have cytotoxic effect. In this way one can provide high confidence that the target cells will be destroyed while providing for the appropriate level of expression of the cytotoxic agents).

Accordingly, the adenovirus vectors of this invention can further include a heterologous polynucleotide (transgene) under the control of a cell type-specific TRE. In this way, various genetic capabilities may be introduced into target cells. For example, in certain instances, it may be desirable to enhance the degree and/or rate of cytotoxic activity, due to, for example, the relatively refractory nature or particular aggressiveness of the target cell. This could be accomplished by coupling the cell-specific replicative cytotoxic activity with cell-specific expression of, for example, HSV-tk and/or cytosine deaminase (cd), which renders cells capable of metabolizing 5-fluorocytosine (5-FC) to the chemotherapeutic agent 5-fluorouracil (5-FU). Using these types of transgenes may also confer a bystander effect.

Genetic capability that may be introduced into the adenovirus vehicle includes a factor capable of initiating apoptosis, antisense or ribozymes, which among other capabilities may be directed to mRNAs encoding proteins essential for proliferation, such as structural proteins, transcription factors, polymerases, etc., viral or other pathogenic proteins, where the pathogen proliferates intracellularly, cytotoxic

proteins, e.g., the chains of diphtheria, ricin, abrin, etc., genes that encode an engineered cytoplasmic variant of a nuclease (e.g., RNase A) or protease (e.g., trypsin, papain, proteinase K, carboxypeptidase, etc.), or encode the Fas gene, and the like. Other genes of interest include cytokines, antigens, transmembrane proteins, and the like, such as IL-1, -2, -6, -12, GM-CSF, G-CSF, M-CSF, IFN-α, -β, -γ, TNF-α, -β, TGF-α, -β, NGF, and the like.

Other opportunities for specific genetic modification include T cells, such as tumor infiltrating lymphocytes (TILs), where the TILs may be modified to enhance expansion, enhance cytotoxicity, reduce response to proliferation inhibitors, enhance expression of lymphokines, etc. One may also wish to enhance target cell vulnerability by providing for expression of specific surface membrane proteins, e.g., B7, SV40 T antigen mutants, etc.

Other desirable transgenes that may be introduced via an adenovirus vector(s) include genes encoding a factor capable of initiating apoptosis, sequences encoding antisense transcripts or ribozymes, which among other capabilities may be directed to mRNAs encoding proteins essential for proliferation, such as structural proteins, or transcription factors; and viral or other pathogenic proteins, where the pathogen proliferates intracellularly. The positive effector genes could be used in an early phase, followed by cytotoxic activity due to replication.

In some embodiments, the adenovirus death protein (ADP), encoded within the E3 region, is maintained (i.e., contained) in the adenovirus vector. The ADP gene, under control of the major late promoter (MLP), appears to code for a protein (ADP) that is important in expediting host cell lysis. Tollefson et al. (1996) *J. Virol.* 70(4):2296; Tollefson et al. (1992) *J. Virol.* 66(6):3633. Thus, adenoviral vectors containing the ADP gene may render the adenoviral vector more potent, making possible more effective treatment and/or a lower dosage requirement.

Accordingly, the invention provides adenovirus vectors in which an adenovirus gene is under transcriptional control of a first cell type-specific TRE and a

polynucleotide sequence encoding an ADP under control of a second cell type-specific TRE, wherein the first and second TREs are substantially identical, and wherein preferably the adenovirus gene is essential for replication. A DNA sequence encoding an ADP and the amino acid sequence of an ADP are depicted in SEQ ID NO:10 and SEQ ID NO:11, respectively. Briefly, an ADP coding sequence is obtained preferably from Ad2 (since this is the strain in which ADP has been more fully characterized) using techniques known in the art, such as PCR. Preferably, the Y leader (which is an important sequence for correct expression of late genes) is also obtained and ligated to the ADP coding sequence. The ADP coding sequence (with or without the Y leader) can then be introduced into the adenoviral genome, for example, in the E3 region (where the ADP coding sequence will be driven by the MLP). The ADP coding sequence could also be inserted in other locations of the adenovirus genome, such as the E4 region. Alternatively, the ADP coding sequence could be operably linked to a different type of TRE, including, but not limited to, another viral TRE.

It is understood that the present invention does not exclude adenovirus vectors containing additional genes under control of cell type-specific TREs. Accordingly, the invention provides adenoviral vectors comprising a third gene under transcriptional control of a third TRE. The third TRE may or may not be substantially identical to the first and second cell type-specific TREs, and the first and second cell type-specific TREs are substantially identical to one another, and all three TREs are functional in the same cell. Preferably, the third gene is one that contributes to cytotoxicity (whether direct and/or indirect), more preferably one that contributes to and/or enhances cell death, and even more preferably the third gene is essential from adenovirus replication. Preferably the third TRE is cell type-specific. For example, an adenovirus vector may contain two *PB*-TREs and an *hKLK2*-TRE, or two *PSE*-TREs and an *hKLK2*-TRE, each prostate cell specific and each controlling the transcription of a different gene.

Accordingly, the invention provides adenoviral vectors comprising at least an additional gene (beyond the first and the second genes) under transcriptional control of a cell type-specific TRE. Preferably, the additional gene is one that contributes to cytotoxicity (whether direct and/or indirect), more preferably one that enhances cell death, and even more preferably the third gene is essential from adenovirus replication.

Delivery of adenoviral vectors to cells

The adenoviral vectors can be used in a variety of forms, including, but not limited to, naked polynucleotide (usually DNA) constructs; polynucleotide constructs complexed with agents to facilitate entry into cells, such as cationic liposomes or other compounds such as polylysine; packaged into infectious adenovirus particles (which may render the adenoviral vector(s) more immunogenic); packaged into other particulate viral forms such as HSV or AAV; complexed with agents to enhance or dampen an immune response; complexed with agents that facilitate *in vivo* transfection, such as DOTMA™, DOTAP™, and polyamines.

If an adenoviral vector is packaged into an adenovirus, the adenovirus itself may be selected to further enhance targeting. For example, adenovirus fibers mediate primary contact with cellular receptor(s) aiding in tropism. See, e.g., Arnberg et al. (1997) *Virol.* 227:239-244. If a particular subgenus of an adenovirus serotype displayed tropism for a target cell type and/or reduced affinity for non-target cell types, such subgenus (or subgenera) could be used to further increase cell-specificity of cytotoxicity and/or cytolysis.

The modified viruses may be delivered to the target cell in a variety of ways, depending upon whether the cells are in culture, *ex vivo* or *in vivo*. For the prostate for the most part, the cells will be delivered *in vivo*. Delivery can be achieved in a variety of ways, employing liposomes, direct injection, catheters, intraveneous inhalation, topical applications, general transfection methods that are well known in the art (such as calcium phosphate precipitation and electroporation), and intravenous

infusion, etc. Due to the high efficiency of transfection of adenoviruses, one can achieve a high level of modified cells. In the case of neoplasia, where toxins are produced, the toxins will be released locally, so as to affect cells which may not have been successfully transfected. In this manner, one can specifically eliminate the neoplastic cells, without significant effect on the normal cells. In addition, expression of adenovirus proteins will serve to activate the immune system against the target cells. Finally, proliferation of the adenovirus in a host cell will lead to cell death. The means of delivery will depend in large part on the particular adenoviral vector (including its form) as well as the type and location of the target cells (i.e., whether the cells are *in vitro* or *in vivo*).

If used in a packaged adenovirus, the adenovirus may be administered in an appropriate physiologically acceptable carrier at a dose of about 10^4 to 10^{11}. The multiplicity of infection will generally be in the range of about 0.001 to 100. The viruses may be administered one or more times, depending upon the immune response potential of the host. If necessary, the immune response may be diminished by employing a variety of immunosuppressants, so as to permit repetitive administration, without a strong immune response.

If administered as a polynucleotide construct (i.e., not packaged as a virus) about 0.01 μg to 1000 μg of an adenoviral vector can be administered. The adenoviral vectors may be administered one or more times, depending upon the intended use and the immune response potential of the host or may be administered as multiple simultaneous injections. If an immune response is undesirable, the immune response may be diminished by employing a variety of immunosuppressants, so as to permit repetitive administration, without a strong immune response. If packaged as another viral form, such as HSV, an amount to be administered is based on standard knowledge about that particular virus (which is readily obtainable from, for example, published literature) and can be determined empirically.

Host Cells and Target Cells

The present invention also provides host cells and target cells comprising (i.e., transformed with) the adenoviral vectors described herein. Host cells include both prokaryotic and eukaryotic host cells as long as sequence requisite for maintenance in that host, such as appropriate replication origin(s), are present. For convenience, selectable markers are also provided. Prokaryotic host include bacterial cells, for example, *E. coli* and mycobacteria. Among eukaryotic host cells are yeast, insect, avian, amphibian, plant and mammalian host cells. Host systems are known in the art and need not be described in detail herein.

Suitable target cells for the adenovirus vectors of the invention include any eukaryotic cell type that allows function of the cell type-specific TREs, preferably mammalian, more preferably human, even more preferably neoplastic cells. Suitable target cells also include any cells that produce proteins and other factors necessary for expression of the gene under control of the cell type-specific TREs, such factors necessary for said expression are produced naturally or recombinantly. For example, if the cell type-specific TRE(s) used is prostate cell-specific, the cells are preferably prostate cells, for example LNCaP cells. The prostate cells used may or may not be producing an androgen receptor, depending on whether the promoter used is androgen-inducible. If an androgen-inducible promoter is used, non-androgen receptor producing cells, such as HLF, HLE, and 3T3 and the non-AR-producing prostate cancer cells PC3 and DU145 can be used, provided an androgen receptor-encoding expression vector is introduced into the cells along with the adenovirus. If the cell type-specific TRE(s) used is derived from the AFP gene, for example, suitable host cells include any cell type that produces AFP, including but not limited to, Hep3B, HepG2, HuH7, HuH1/C12. Activity of a given TRE in a given cell can be assessed by measuring the level of expression of a operably-linked reporter gene using standard assays. The comparison of expression between cells in which the TRE is suspected of being functional and the control cell indicates the presence or absence of transcriptional enhancement.

Comparisons between or among various TREs can be assessed by measuring and comparing levels of expression within a single target cell line. It is understood that absolute transcriptional activity of a TRE will depend on several factors, such as the nature of the target cell, delivery mode and form of a TRE, and the coding sequence that is to be selectively transcriptionally activated. To compensate for various plasmid sizes used, activities can be expressed as relative activity per mole of transfected plasmid. Alternatively, the level of transcription (i.e., mRNA) can be measured using standard Northern analysis and hybridization techniques. Levels of transfection (i.e., transfection efficiencies) are measured by co-transfecting a plasmid encoding a different reporter gene under control of a different TRE, such as the CMV immediate early promoter. This analysis can also indicate negative regulatory regions, i.e., silencers.

Compositions

The present invention also includes compositions, including pharmaceutical compositions, containing the adenoviral vectors described herein. Such compositions are useful for administration *in vivo*, for example, when measuring the degree of transduction and/or effectiveness of cell killing in an individual. Preferably, these compositions further comprise a pharmaceutically acceptable excipient. These compositions, which can comprise an effective amount of an adenoviral vector of this invention in a pharmaceutically acceptable excipient, are suitable for systemic administration to individuals in unit dosage forms, sterile parenteral solutions or suspensions, sterile non-parenteral solutions or oral solutions or suspensions, oil in water or water in oil emulsions and the like. Formulations for parenteral and nonparenteral drug delivery are known in the art and are set forth in *Remington's Pharmaceutical Sciences*, 18th Edition, Mack Publishing (1990). Compositions also include lyophilized and/or reconstituted forms of the adenoviral vectors (including those packaged as a virus, such as adenovirus) of the invention.

Other compositions are used, and are useful for, detection methods described herein. For these compositions, the adenoviral vector usually is suspended in an appropriate solvent or solution, such as a buffer system. Such solvent systems are well known in the art.

Kits

The present invention also encompasses kits containing an adenoviral vector of this invention. These kits can be used for diagnostic and/or monitoring purposes, preferably monitoring. Procedures using these kits can be performed by clinical laboratories, experimental laboratories, medical practitioners, or private individuals. Kits embodied by this invention allow one to detect the presence of target cells in a suitable biological sample, such as biopsy specimens.

The kits of the invention comprise an adenoviral vector described herein in suitable packaging. The kit may optionally provide additional components that are useful in the procedure, including, but not limited to, buffers, developing reagents, labels, reacting surfaces, means for detection, control samples, instructions, and interpretive information.

Preparation of the adenovirus vectors of the invention

The adenovirus vectors of this invention can be prepared using recombinant techniques that are standard in the art. Generally, cell type-specific TREs are inserted 5' to the adenoviral genes of interest, preferably one or more early genes (although late gene(s) may be used). Cell type-specific TREs can be prepared using oligonucleotide synthesis (if the sequence is known) or recombinant methods (such as PCR and/or restriction enzymes). Convenient restriction sites, either in the natural adeno-DNA sequence or introduced by methods such as PCR or site-directed mutagenesis, provide an insertion site for the cell type-specific TREs. Accordingly, convenient restriction sites for annealing (i.e., inserting) cell type-specific TREs can be engineered onto the 5' and 3' ends of the cell type-specific TRE using standard recombinant methods, such as PCR.

Polynucleotides used for making adenoviral vectors of this invention may be obtained using standard methods in the art such as chemical synthesis recombinant methods and/or obtained from biological sources.

The vectors are conveniently prepared by employing two plasmids, one plasmid providing for the left-hand region of adenovirus and the other plasmid providing for the right hand region, where the two plasmids share at least about 500nt of middle region for homologous recombination. In this way, each plasmid, as desired, may be independently manipulated, followed by cotransfection in a competent host, providing complementing genes as appropriate, or the appropriate transcription factors for initiation of transcription from the PSE for propagation of the adenovirus.

For convenience, plasmids are available that provide the necessary portions of the adenovirus. Plasmid pXC.1 (McKinnon (1982) *Gene* 19:33-42) contains the wild-type left-hand end of Ad5. pBHG10 provides the right-hand end of Ad5, with a deletion in E3. The deletion in E3 provides room in the virus to insert the 2kb minimal PSE without deleting the wild-type enhancer-promoter. The gene for E3 is located on the opposite strand from E4 (r-strand).

For manipulation of the early genes, the transcription start site of Ad5 ElA is at nt 560 and the ATG start site of the ElA protein is at nt 610 in the virus genome. This region can be used for insertion of the cell specific element, *e.g.*, PSE. Conveniently, a restriction site may be introduced by employing the polymerase chain reaction (PCR), where the primer that is employed may be limited to the Ad5 genome, or may involve a portion of the plasmid carrying the Ad5 genomic DNA. For example, where pBR322 is the backbone, the primers may use the EcoRI site in the pBR322 backbone and the Xpal site at nt 1339 of Ad5. By carrying out the PCR in two steps, where overlapping primers at the center of the region introduce a sequence change resulting in a unique restriction site, one can provide for insertion of the cell specific response element at that site.

A similar strategy may also be used for insertion of the cell specific response element to regulate ElB. The ElB promoter of Ad5 consists of a single high-affinity recognition site for Spl and a TATA box. This region extends from 1636 to 1701 nt. By insertion of the cell specific response element in this region, one can provide for cell specific transcription of the ElB gene. By employing the left-hand region modified with the cell specific response element regulating ElA, as the template for introducing the cell specific response element to regulate ElB, the resulting adenovirus will be dependent upon the cell specific transcription factors for expression of both ElA and E1B.

For example, we have introduced an AgeI site 12 bp 5' to the E1A initiation codon (Ad5 nucleotide 547) by oligo-directed mutagenesis and linked PCR. In addition, an EagI site was created upstream of the ElB start site by inserting a G residue at Ad5 nt 1682 by oligonucleotide directed mutagenesis. To simplify insertion of a TRE in the EagI site, the endogenous EagI site in CN95 was removed by digestion with EagI, treatment with mung bean nuclease, and religation to construct CN114. In this way, we generated an adenovirus vector containing unique AgeI and EagI sites in the proximal upstream region to E1A and E1B, respectively. Using these unique sites, one can insert a TRE which has engineered AgeI or EagI sites, thus simplifying construction of recombinant adenovirus vectors. Accordingly, the invention includes an adenoviral vector comprising a unique AgeI site 5' of the E1A initiation codon and a unique EagI site 5' of E1B.

For E4, one must use the right-hand portion of the adenovirus genome. The E4 transcription start site is predominantly at nt 35605, the TATA box at nt 35631 and the first AUG/CUG of ORF1 is at nt 35532 (Virtanen et al. (1984) *J. Virol.* 51:822-831). Using any of the above strategies for the other genes, the cell specific response element may be introduced in the region between the transcription start site and the initiation codon. Once again, by employing a previously manipulated adenovirus genome, one can provide for a plurality of genes being dependent upon

the target cell specific transcription factor, insuring that the adenovirus will be incapable of replication in cells lacking these transcription factors.

Similarly, a cell type-specific TRE may be inserted upstream of the E2 gene to make its expression cell type-specific. The E2 early promoter, mapping in Ad5 from 27050-27150, consists of a major and a minor transcription initiation site, the latter accounting for about 5% of the E2 transcripts, two non-canonical TATA boxes, two E2F transcription factor binding sites and an ATF transcription factor binding site (for a detailed review of the E2 promoter architecture see Swaminathan et al., *Curr. Topics in Microbiol. and Immunol.* (1995) 199 part 3:177-194).

The E2 late promoter overlaps with the coding sequences of a gene encoded by the counterstrand and is therefore not amenable to genetic manipulation. However, the E2 early promoter overlaps only for a few base pairs with sequences coding for a 33-kD protein on the counterstrand. Notably, the SpeI restriction site (Ad5 position 27082) is part of the stop codon for the above mentioned 33 kD protein and conveniently separates the major E2 early transcription initiation site and TATA-binding protein site from the upstream transcription factor biding sites E2F and ATF. Therefore, insertion of a cell type-specific TRE having SpeI ends into the SpeI site in the plus-strand would disrupt the endogenous E2 early promoter of Ad5 and should allow TRE regulated expression of E2 transcripts.

For E4, one must use the right hand portion of the adenovirus genome. The E4 transcription start site is predominantly at nt 35609, the TATA box at nt 35638 and the first AUG/CUG of ORF1 is at nt 35532. Virtanen et al. (1984) *J. Virol.* 51: 822-831. Using any of the above strategies for the other genes, a heterologous TRE may be introduced upstream from the transcription start site. For the construction of mutants in the E4 region, the co-transfection and homologous recombination are performed in W162 cells (Weinberg *et al.* (1983) *Proc. Natl. Acad. Sci. USA* 80:5383–5386) which provide E4 proteins *in trans* to complement defects in synthesis of these proteins.

Methods of packaging adenovirus polynucleotides into adenovirus particles are known in the art and are described in the Examples.

Methods using the adenovirus vectors of the invention

The subject vectors can be used for a wide variety of purposes, which will vary with the desired or intended result. Accordingly, the present invention includes methods using the adenoviral vectors described above. In one embodiment, methods for using adenovirus vectors comprise introducing an adenovirus vector into a cell, preferably a eukaryotic cell, more preferably a mammalian cell.

Purposes for introducing transient expression include indications that may be treated involving undesired proliferation other than tumors, such as psoriatic lesions, restenosis, wound healing, tissue repair, enhanced immune response, resistance to infection, production of factors, enhanced proliferation, investigation of metabolic or other physiological pathways, comparison of activity of cells in the presence and absence of the adenovirus introduced transgene, by comparing the activity of the cell before, during and after the modification with the adenovirus, etc. The subject vectors can be used to free a mixture of cells of a particular group of cells, where the group of cells is the target cells. By having the adenovirus be selectively competent for propagation in the target cells, only those cells will be killed on proliferation of the adenovirus. By combining the adenovirus with the mixture of cells, for example, in culture or *in vivo*, the adenovirus will only be capable of proliferation in the target cells. In this way cells other than the target cells will not be affected by the adenovirus, while the target cells will be killed. The expansion of the adenovirus due to propagation in the target cells will ensure that the mixture is substantially freed of the target cells. Once the target cells are destroyed, the adenovirus will no longer be capable of propagation, but in culture may be retained so as to continually monitor the mixture for recurrence of the target cell, e.g., a mutated cell or neoplastic cell.

By identifying genes that are expressed specifically by the target host cells, based on the nature of the cells, their level of maturity or their condition, the target cell specific response element can be used to provide genetic capability to such cells, where the genetic capability will be absent in other cells, even when transfected with the adenovirus vehicle.

In one embodiment, methods for using adenovirus vectors comprise introducing an adenovirus vector into a target cell, preferably a neoplastic cell. In another embodiment, methods for using adenovirus vectors comprise introducing an adenovirus vector into a prostate cell. In another embodiment, methods for using adenovirus vectors comprise introducing an adenovirus vector into a liver cell. In another embodiment, methods for using adenovirus vectors comprise introducing an adenovirus vector into a breast cancer cell. In another embodiment, methods for using adenovirus vectors comprise introducing an adenovirus vector into a colon cancer cell.

In one embodiment, methods are provided for conferring selective cytotoxicity in cells which allow function of the cell type-specific TRE, comprising contacting cells with an adenovirus vector described herein, such that the adenovirus vector(s) enters, i.e., transduces the cell(s). Cytotoxicity can be measured using standard assays in the art, such as dye exclusion, ^{3}H-thymidine incorporation, and/or lysis.

In another embodiment, methods are provided for propagating an adenovirus specific for cells which allow function of the cell type-specific TRE(s), preferably eukaryotic cells, more preferably mammalian cells. These methods entail combining an adenovirus vector with mammalian cells which allow function of the cell type-specific TREs, whereby said adenovirus is propagated.

Another embodiment provides methods of killing cells that allow a cell type-specific TRE to function (i.e., target cells) comprising combining the mixture of cells with an adenovirus vector of the present invention. The mixture of cells is generally

a mixture of cancerous cells in which the cell type-specific TREs are functional and normal cells, and can be an *in vivo* mixture or *in vitro* mixture.

The invention also includes methods for detecting cells in which a cell type-specific TRE is functional in a biological sample. These methods are particularly useful for monitoring the clinical and/or physiological condition of an individual (i.e., mammal), whether in an experimental or clinical setting. For these methods, cells of a biological sample are contacted with an adenovirus vector, and replication of the adenoviral vector is detected. A suitable biological sample is one in which target cells may be or are suspected to be present. Generally, in mammals, a suitable clinical sample is one in which target cancerous cells are suspected to be present. Such cells can be obtained, for example, by needle biopsy or other surgical procedure. Cells to be contacted may be treated to promote assay conditions such as selective enrichment and/or solubilization. In these methods, target cells can be detected using *in vitro* assays that detect proliferation, which are standard in the art. Examples of such standard assays include, but are not limited to, burst assays (which measure virus yields) and plaque assays (which measure infectious particles per cell). Also, propagation can be detected by measuring specific adenoviral DNA replication, which are also standard assays.

The invention also provides methods of modifying the genotype of a target cell, comprising contacting the target cell with an adenovirus vector described herein, wherein the adenoviral vector enters the cell.

The invention further provides methods of suppressing tumor cell growth, comprising contacting a tumor cell with an adenoviral vector of the invention such that the adenoviral vector enters the tumor cell and exhibits selective cytotoxicity for the tumor cell. Tumor cell growth can be assessed by any means known in the art, including, but not limited to, measuring tumor size, determining whether tumor cells are proliferating using a ^{3}H-thymidine incorporation assay, or counting tumor cells. "Suppressing" tumor cell growth means any or all of the following states: slowing,

delaying, and stopping tumor growth, as well as tumor shrinkage. "Suppressing" tumor growth indicates a growth state that is curtailed when compared to growth without contact with, i.e., transfection by, an adenoviral vector described herein. See Example 3, Figure 6.

The invention also provides methods of lowering the levels of a tumor cell marker in an individual, comprising administering to the individual an adenoviral vector of the present invention, wherein the adenoviral vector is selectively cytotoxic in cells producing the tumor cell marker. Tumor cell markers include, but are not limited to, PSA, CEA and hK2. Methods of measuring the levels of a tumor cell marker are known to those of ordinary skill in the art and include, but are not limited to, immunological assays, such as enzyme-linked immunosorbent assay (ELISA), using antibodies specific for the tumor cell marker. In general, a biological sample is obtained from the individual to be tested, and a suitable assay, such as an ELISA, is performed on the biological sample. See Example 3, Fig. 7.

The invention also provides methods of treatment, in which an effective amount of an adenoviral vector(s) described herein is administered to an individual. For example, treatment using an adenoviral vector(s) in which at least one cell type-specific TRE is specific for prostate cells (e.g., *PSE*-TRE, *PB*-TRE, and/or *hKLK2*-TRE) is indicated in individuals with prostate-associated diseases as described above, such as hyperplasia and cancer. In this example, also indicated are individuals who are considered to be at risk for developing prostate-associated diseases, such as those who have had disease which has been resected and those who have had a family history of prostate-associated diseases. Determination of suitability of administering adenoviral vector(s) of the invention will depend, inter alia, on assessable clinical parameters such as serological indications and histological examination of tissue biopsies. Generally, a pharmaceutical composition comprising an adenoviral vector(s) is administered. Pharmaceutical compositions are described above.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

EXAMPLE 1

Replication Competent Prostate-Specific Attenuated Adenoviruses

Replication-competent adenoviral vectors were constructed in which a PSE mediates transcription of at least one adenoviral gene.

1. Ad5 with PSE Driving Expression of E1A

The cloning and characterization of a minimal prostate-specific enhancer (PSE) is described in Prostate Specific Antigen Expression is Regulated by an upstream Enhancer (Schuur et al., *supra*). Plasmid CN71 contains our minimal PSE (from-5322 bp to -3875bp relative to the transcription start site of the PSA gene) and -532 to +11 of the PSA promoter. CN71 was cut with XhoI/HindIII which removes the PSA promoter. A shorter promoter, from -230 to +7, amplified by PCR using primers:

18.119, 5' -GGACCTCGAGGTCTCCATGAGCTAC, (SEQ ID NO:12) and

15.59B, 5' -AGCTCGAGCTTCGGGATCCTGAG (SEQ ID NO:13).

The PCR product was cut with XhoI/HindIII and ligated back into XhoI/HindIII cut CN71 creating CN105.

1A. Attenuated Ad5 with PSE Driving E1A and Retaining the Endogenous Ad5 E1A Promoter and Enhancer

The E1A gene is expressed immediately after viral infection (0-2 hours) and before any other viral genes. The E1A protein acts as a trans-acting, positive-acting transcriptional regulatory factor required for the expression of the other early viral genes, E1B, E2, E3, E4, and the promoter proximal genes of the major late genes.

Despite the nomenclature, the promoter proximal genes driven by the major late promoter are expressed during early times after Ad5 infection (Flint (1982) *Biochem. Biophys. Acta* **651**:175-208; Flint (1986) *Advances Virus Research* **31**:169-228; Grand (1987) *Biochem. J.* **241**:25-38). In the absence of a functional ElA gene, viral infection does not proceed for the gene products necessary for viral DNA replication are not produced (Nevins (1989) *Adv. Virus Res.* **31**:35-81). The transcription start site of Ad5 ElA is at nt 560 and the ATG start site of the E1A protein is at nt 610 in the virus genome.

pXC.1 was purchased from Microbix Biosystems Inc. (Toronto). pXC.1 contains Adenovirus 5 sequences from bp22 to 5790. We have introduced an AgeI site 12 bp 5' to the E1A initiation codon (Ad5 nucleotide 547) by oligo-directed mutagenesis and linked PCR. The plasmid pXC.1 was PCR amplified using primers:

15.133A, 5'-TCGTCTTCAAGAATTCTCA (SEQ ID NO:14), containing an EcoRI site, and

15.134B, 5'-TTTCAGTCACCGGTGTCGGA (SEQ ID NO:15), containing an extra A to introduce an AgeI site. This created a segment from the EcoRI site in the pBR322 backbone to Ad5 nt 560. A second segment of pXC.1 from Ad nucleotide 541 to the XbaI site at Ad nucleotide 1339 was amplified using primers:

15.133B, 5'-GCATTCTCTAGACACAGGTG (SEQ ID NO:16) containing an Xbal site, and

15.134A, 5' -TCCGACACCGGGTGACCTGAAA (SEQ ID NO:17), containing an extra T to introduce an AgeI site. A mixture of these two PCR amplified DNA segments was mixed and amplified with primers 3 and 4 to create a DNA segment from the EcoRI site to the XbaI site of pXC.1. This DNA segment encompasses the leftmost 1317 bases of Adenovirus sequence and contained an AgeI site at Ad nucleotide 547. This DNA segment was used to replace the corresponding

segment of pXC.1 to create CN95. Similarly, a PSE with AgeI ends was PCR amplified from CN105 using primers:

15.176A, 5'-CATTAACCGGTACCTCTAGAAAATCTAGC (SEQ ID NO:18) and

15.176B, 5'-CATTAACCGGTAAGCTTGGGGCTGGGG (SEQ ID NO:19) and cloned into CN95. The virus created by homologous recombination of CN96 and BHG10 was designated CN706.

1B. Attenuated Ad5 with PSE Driving Ad5 ElA Deleted for the Ad5 Endogenous Promoter and Enhancer

In order to reduce ubiquitous expression of the ElA gene we decided to delete the endogenous E1A transcription regulatory DNA sequences. The transcriptional regulatory sequences of the ElA gene are intricately embedded in DNA sequences essential for DNA packaging (see Graeble and Hearing (1992) and References cited therein). Graeble and Hearing (1990) have shown that an Adenovirus 5 with a deletion from bp 194 to bp 316 which eliminates all transcriptional regulatory elements and retains only three out of seven packaging signals reduced the yield only 3-fold compared to wild type. These observations suggested that the ElA transcription regulatory sequences are dispensable and the loss of the first three out of seven packaging signals allowed virus production in acceptable quantities.

a. In the first variant, the region of the Ad5 genome containing the ElA enhancer and promoter and the Ad5 packaging sequence were deleted and replaced with a synthetic DNA segment containing a mutated packaging sequence and a PCR amplified segment of the PSE from CN127. In this construction the EcoRI/XbaI fragment of pXC.1 containing the first 1339 bases of the Ad5 genome was cloned into pUC19 to construct CN172 as a substrate for further manipulations. The DNA sequences corresponding to Ad5 nt 123 to nt 497 were deleted from CN172 by PCR amplification using primers:

26.153.1, 5'-CCGCTCGAGATCACACTCCGCCACAC (SEQ ID NO:20) containing an XhoI site, and

26.153.2, 5'-CCGCTCGAGCACTCTTGAGTGCCA (SEQ ID NO:21), containing an XhoI site. Cleavage of the PCR product with XhoI followed by religation resulted in CN178 in which an XhoI site replaced Ad5 nt 123 to 497. The synthetic DNA segment containing the mutated Ad5 packaging sequences was composed of the following two strands:

26.160.1:5'-

TCGAGGGATGTTGTAGTAAATTTGGGCGTAACCGAGTAAGATTTGGCCATTTTCGCGGGAAAACTGAA
TAAGACTCTTCGAAATCTGAATAATTTTGTGTTACTCATAGCGCGTAATATTTGTCTAGGGCCGCGGG
GACTTTGACCGTTTACGTGG (SEQ ID NO:22)

26.160.2:5'-

GATCCCACGTAAACGGTCAAAGTCCCCGCGGCCCTAGACAAATATTACGCGCTATGAGTAACACAAAA
TTATTCAGATTTCGAAGAGTCTTATTCAGTTTTCCCGCGAAAATGGCCAAATCTTACTCGGTTACGCC
CAAATTTACTACAACATCCC (SEQ ID NO:23)

The strands were annealed and kinased using T4 polynucleotide kinase to form the dsDNA and allow ligation to the other DNA segments in the construct.

The PSE segment used for ligation was PCR amplified from CN127 using primers:

26.160.3, 5'-GGAAGATCTGAAATCTAGCTGATATAG (SEQ ID NO:24), containing an XhoI site, and

19.16.5,5'-TTCTCGAGAAGCTTGGGGCTGGGG (SEQ ID NO:25), containing XhoI and HinDIII sites. For ligation, the PSE PCR product and CN178 were both cleaved with XhoI. The XhoI cut CN178, XhoI cut PSE PCR product, and the kinased packaging oligonucleotide were mixed in equal molar ratios and ligated with T4 DNA ligase. The resulting recombinant was designated CN201. The EcoRI/XbaI segment of CN201 containing the mutated packaging sequence and PSE driving ElA was excised from CN201 and used to replace the homologous segment of pXC.1 to generate CN202.

b. In the second variant, a different strategy was employed. In order to perform the deletion mutagenesis with a relatively small plasmid, a 2297 bp EcoRI-XhoI fragment of plasmid CN145, which contains the left end Adeno sequences including the ElA promoter region and the PSA enhancer, was subcloned into similarly cut pBluescript SKII+ yielding plasmid CN169.

The plan for the deletion mutagenesis was to delete the sequences from Ad position 194-301 and replace them with a SalI restriction site 5'-GTCGAC-3' which served as diagnostic marker to distinguish mutagenized plasmids from parental plasmids. The deletion eliminated all ElA core and E2F transcription regulatory elements as well as packaging signals AI and AII, but will preserve packaging signals AIII, AIV, AV, AVI and AVII. To this end, two oligonucleotide primers were synthesized:

28.134A, 5'-GTCGACGTGAAATCTGAATAATTTTGTGTTACTCATAGC (SEQ ID NO:26). This primer matches to sequences 302-334 in Ad5.

28.134B, 5'-CACCGGCGCACACCAAAAACGTC (SEQ ID NO:27). This primer matches to sequences 171-193 in Ad5.

The PCR mutagenesis kit from Stratagene was used in the following manipulations. In a PCR tube, 15 pMol of each primer was added to 0.5 pMol CN169; 1 mM dNTP, 2.5 µl 10 x PCR 11 (Stratagene), dH_2O to 24 µl and 0.5 µl each of Taq Polymerase and TaqExtender (Stratagene). The mixture was overlaid with 20 µl mineral oil and programmed for PCR: 94°C 4 minutes, 63°C 1 minute, 72°C 4 minutes for cycle and 94°C 1 minute, 63°C 1 minute, 72°C 4 minutes for 10 cycles. l µl Dpn I restriction enzyme (Stratagene) was added to cut parental DNA and incubated at 37°C for 80 minutes followed by the addition of 1 µl Pfu Polymerase (Stratagene) and incubation at 72°C for 50 minutes to fill up protruding DNA ends generated during the former PCR process by the Taq polymerase. The PCR yielded a 5 kb linear DNA which was ligated with T4 DNA ligase to recircularize. XL-1 bacteria were transformed with the ligation reaction and mutagenized recombinants

were identified by virtue of the presence of the unique SalI restriction site. One of the recombinants, CN 179, was used to rebuild the parental plasmid CN145 with the deletion by swapping the EcoRI-XhoI fragment of CN145 containing the Adeno-and PSE sequences with the one of CN179, yielding plasmid CN185. Plasmid CN185 was used in cotransfections with BHG11 into human 293 cells to generate recombinant Adenoviruses. Nine virus plaques were isolated. One virus isolate was designated CN724.

2. Attenuated Ad5 with PSE Driving Expression of ElB

The ElB protein functions in trans and is necessary for transport of late mRNA from the nucleus to the cytoplasm. Defects in ElB expression also results in poor expression of late viral proteins and an inability to shut off host-cell protein synthesis. The promoter of ElB has been implicated as the defining element of difference in the host range of Ad40 and Ad5: clinically Ad40 is an enterovirus, whereas Ad5 causes acute conjunctivitis (Bailey, Mackay et al. (1993) *Virology* **193**:631; Bailey et al. (1994) *ibid* **202**:695-706). The ElB promoter of Ad5 consists of a single high-affinity recognition site for Spl and a TATA box.

To insert a PSE driving expression of ElB in Ad5, an EagI site was created upstream of the ElB start site by inserting a G residue at Ad5 nt 1682 by oligonucleotide directed mutagenesis as above. To simplify insertion of the PSE in the EagI site the endogenous EagI site in CN95 was removed by digestion with EagI, treatment with mung bean nuclease, and religation to construct CN114. The primers:

15.133A, 5'-TCGTCTTCAAGAATTCTCA (SEQ ID NO:14), containing an EcoRI site, and

9.42, 5'-GCCCACGGCCGCATTATATAC (SEQ ID NO:28), containing an extra C, were used to amplify the segment between the EcoRI site and Ad5 nt 1682.

Primers:

9.39, 5'-GTATATAATGCGGCCGTGGGC (SEQ ID NO:29) containing an extra G, and

24.020, 5'-CCAGAAAATCCAGCAGGTACC (SEQ ID NO:30), containing a KpnI site, were used to amplify the segment between 1682 and the KpnI site at Ad5 nt 2048. Co-amplification of the two segments with primers 9 and 12 yields a fragment with an EagI site at Ad5 nt 1682 which was used to replace the corresponding EcoRI /KpnI site in pXC.1 to construct CN124. PSE amplified from CN105 with primers:

26.1.1, 5'-TAACGGCCGTCTAGAAATCTAGCTGA (SEQ ID NO:31) and

26.1.2, 5'-TAACGGCCGAAGCTTGGGCTGGG (SEQ ID NO:32), with EagI ends, was ligated into the EagI site of CN124 to construct CN125. The resultant virus from homologous recombination of CN125 and BHG10 was designated CN711.

3. Attenuated Ad5 with PSE Driving Expression of Both ElA and ElB

A left end Ad5 plasmid with the PSE driving expression of both ElA and ElB was constructed by PCR amplifying CN95 with primers 9-12 as described for the construction of CN124. The resulting DNA segment contains the AgeI site derived from CN95 and the EagI site derived from the PCR mutagenesis. This DNA segment was cloned back into CN114 (the plasmid from which the EagI site was removed from pXC.1) to construct the plasmid CN144. CN144 contains a single AgeI site at Ad5 nt 547, and a single EagI site at Ad5 nt 1682. PSE segments were PCR-amplified with AgeI ends from CN105 or EagI ends, also by PCR from CN105, as described above and ligated into the appropriate sites of CN144 to construct CN145. CN145 is a plasmid in which the PSE drives expression of both ElA and ElB while retaining the Ad5 endogenous promoters and enhancers of both genes. Clones with the PSE in the left to right orientation were chosen. The endogenous Ad5 ElA and ElB promoter/enhancers were moved upstream by insertion of both PSE segments.

The resultant virus derived by homologous recombination of CN145 and BHG10 was designated CN716.

4. Attenuated Ad5 with PSE Driving Expression of E4

E4 is located at the far right-hand end of the Ad5 genome and read right-to left from the 1-stand (Flint, *supra*). E4 can be deleted from the Ad5 genome and supplied in trans by W162 cells, a derivative of VERO cells (Weinberg and Ketner, *supra*). The transcription products of E4 are complex. Open-reading frames (ORF) 3 and ORF 6 of the E4 transcription unit increase the accumulation of major late transcription unit mRNAs by binding the 55-kDa protein from E1B (Dix and Leppard (1993) *J. Virol.* **67**:3226-3231) and heterodimers of E2F-1 and DP-1 (Helin and Harlow (1994) *J. Virol.* **68**:5027-5035). Mutations such that neither ORF 3 nor ORF 6 encode functional proteins, produce plaques with an efficiency less than 10^{-6} that of wild-type virus (Bridge and Ketner (1989) *J. Virol.* **67**:5911-5921).

To facilitate insertion of the PSE driving E4 expression, the 10 kb EcoRI fragment of BHG10 containing the 3' 8 kb of Ad5 plus a portion of the pBR322 backbone was cloned into the EcoRI site of Bluescript KSII+ to construct CN108. A DraIII site at Ad nt 33906 was eliminated by partial digestion of CN108, endfilling with Klenow, and relegation to construct CN113. An XhoI site was introduced at Ad nt 35577 by oligonucleotide directed mutagenesis and linked PCR as described above using primers:

10.1, 5'-TAACTCACGTTGTGCATTGT (SEQ ID NO:33), containing a DraII site,

10.4, 5'-GGTGCCGTGCTCGAGTGGTGT (SEQ ID NO:34), containing an extra C,

10.3, 5'-ACACCACTCGAGCACGGCACC (SEQ ID NO:35), containing an extra G,

19.158, 5'-GCTACTATTCGACAGTTTGTACTG (SEQ ID NO:36), containing a ClaI site.

The PCR product containing an XhoI site as well as DraIII and ClaI ends was used to replace the corresponding DraIII/ClaI fragment of CN113 to construct CN122.

Plasmid CN70 contains the minimal PSE (from-5322 bp to -4023 bp relative to the transcription start site of the PSA gene) and -532 to + 11 of the PSA promoter. CN70 was cut with XhoI/HindIII which removes the PSA promoter. A shorter promoter, from -230 to +7, amplified by PCR using primers:

18.119, 5'-GGACCTCGAGGTCTCCATGAGC TAC (SEQ ID NO:12), and

15.59B, 5'-AGCTCGAGCTTCGGGATCCTGAG (SEQ ID NO:13), was ligated in it's place to construct CN104. CN127 was constructed from CN104 as follows: CN104 was cut with XhoI, end-filled with Klenow, and relegated to remove the XhoI site. The PSE from CN127 was PCR amplified using primers:

19.16.1, 5'-GGGTCGACGTACCTCTAGAAATCTAGC (SEQ ID NO:37) and

19.16.5, 5'-TTGTCGACAAGCTTGGGGCTGGGG (SEQ ID NO:25), to create SalI ends. This DNA segment was then ligated to XhoI cut CN122 to insert the PSE in the correct orientation upstream of E4. The resulting plasmid was designated CN135. The kanamycin resistance gene from pABS4 (Microbix) was inserted into CN135 at the PacI site to construct CN146; the EcoRI fragment of CN146 (containing the adenovirus sequences with the inserted PSE and kanamycin resistance gene) was then ligated to the large EcoRI fragment of BHG10, replacing the homologous wild type Ad sequences in BHG10. Recombinants were identified by resistance to both ampicillin and kanamycin, then the kanamycin gene was excised by PacI digestion and relegation to yield CN190 which is BHG10 with the PSE inserted upstream of the E4 coding region.

5. Attenuated Ad5 with PSE Driving Ad5 EIA containing Cytosine Deaminase in ΔE3

A prostate specific adenovirus vector that contains the cytosine deaminase ("cd") gene incorporated into its genome could deliver this gene to targeted tissue (i.e. prostate tumors). Consequently, infected cancer cells would metabolize 5-FC and release the chemotherapeutic agent 5-FU into the surrounding tissue suppressing cell division, and exhibit the so-called "bystander effect" (Hirshowitz et al. (1995) *Human Gene Ther.* **6**:1055-1063; Griffith and Jarvis (1993) *J. Biol. Chem.* **268**:20085-20090). In contrast, noninfected, nonproximal cells would not be affected. This suggests two uses for the cd gene in an attenuated adenovirus vector. First, cd can serve as an additional therapeutic agent to provide a bystander killing ability and expedite local tumor reduction without systemic toxicity (Moolten and Wells (1990) *J. Nat'l Cancer Inst.* **82**:297-300). Second, the gene can serve as a recall mechanism to halt a runaway infection by preventing viral DNA and RNA synthesis in infected and noninfected, local cells.

The enzyme cytosine deaminase, which deaminates cytosine to uracil, is found in many bacteria and fungi. These microorganisms can convert 5-fluorocytosine (5-FC), a harmless prodrug, to 5-fluorouracil (5-FU), a highly toxic compound that inhibits both DNA and RNA synthesis (Calibrisi and Chabner *Goodman and Gilman's The Pharmacological Basis of Therapeutics* (Eds. A.G. Gilman, T. Rall, A.S. Nies, and P. Taylor, Pergamon, NY) (1990) 8 ed., pp 1209-1263); Damon et al. (1989). Because mammalian cells do not express significant amounts of the cd gene, they are not sensitive to 5-FU. Mammalian cells modified by gene transfer to express the gene can metabolize 5-FC, however. In this application, cd acts as a "suicide gene" selectively conferring sensitivity to those cells that contain the gene.

Adenovirus Vector Construction. The plasmid pCMV-cd, which contains cd coding region downstream of the CMV promoter, was obtained from David Crooks

(Stanford). A SpeI restriction endonuclease site located in a multiple cloning region between the promoter and the cd ATG was removed by digesting the plasmid with enzymes which recognize sequences flanking the SpeI site, BamHI and EcoRI, filling the ends with Klenow, and relegating (CN130). With this site removed, the CMV-cd cassette was cloned by digesting CN130 with SpeI and ligating the appropriate fragment into the XbaI site in pABS4 (Microbix, Toronto), a shuttle plasmid containing the kanamycin-resistance gene (CN131). By digesting CN131 with PacI, a fragment containing the Kan^R gene and the cd gene was isolated and ligated into similarly cut BHG11 (Microbix), which contains a unique PacI site engineered in the E3 region of Ad5 (CN141). The kan^R gene was removed by digesting CN141 with SwaI and religating the vector (CN148).

Two Ad5 recombinant viruses containing the cd gene in the E3 region were constructed. The first contains only the CMV-cd cassette in the E3 region (CN719). The second has the CMV-cd cassette in E3 and the prostate specific enhancer (PSE) minimal element modulating expression of EIA proteins (CN720). Viruses were generated by homologous recombination in low passage 293 cells, a human kidney cell line that expresses Ad EIA and EIB proteins, accomplished by cotransfecting them with pXCl/CN148 and CN145(PSE-EIA)/CN148.

In vitro Characterization. In this first functional assay, CN720, an attenuated, prostate-specific adenovirus containing the cd gene in the E3 region, was studied to test its ability to confer 5-FC sensitivity on infected cells and neighboring cells. Wild type Ad5 (CN702) was also tested. CV1 cells, a semipermissive monkey kidney cell line, seeded in four, 96 well microtitre plates in DMEM, 5 % FBS, were infected in a series of 1:2 dilutions from wells 1-11 with either CN702 or CN720. The multiplicity of infection of well one was approximately twenty-five for CN702 and two for CN720. Row 12 in each plate was left as an noninfected control. One day post infection the media was changed. Two plates of cells, one infected with CN720 and one infected with CN702, were treated with 5 mM 5-FC. The media on the

remaining two plates was changed with complete DMEM only. These infected, untreated cells illustrate the lytic ability of the virus and were used to differentiate between the two causes of cell death in this experiment, virus cell lysis and 5-FU toxicity. The cells were fixed with 50% methanol-50% acetone and stained with Giemsa stain 6 days after the prodrug was administered. Plates were assayed by measuring absorbance at 530 nm in a SpectraMAX 340 microtitre plate reader (Molecular Devices). Cell survival was calculated by relating the absorbance of the cells in the noninfected wells to the absorbance in infected wells. The results were graphed as cell survival versus virus dilution.

Several conclusions can be made from this experiment. Most important, the graph suggests that the recombinant adenoviruses are expressing the cd gene. While the cell killing ability of both viruses appears to increase in the presence of 5-FC, perhaps due to a generalized toxicity to high concentrations of the prodrug, the change in cell killing is dramatic for CN720. The graph of CN720 shows a clear cell survival difference between 5-FC treated cells and untreated cells indicative of a 5-FU bystander effect. This result illustrates the potential to exploit cd function to either enhance the killing potential of Ad5 or to harness a runaway infection by generating an intracellular pool of toxic drug in noninfected cells that prevents DNA replication, a recall mechanism.

As an *in vitro* model, six 96 well plates were seeded with a human intestine epithelia cell line, DLD-1, that is permissive to human Ad in DMEM, 10% FBS. They were infected as described above with Ad5-cd virus (CN719). Prodrug (l mM) was added to one plate at each time point, 0 hrs, 24 hrs, and 48 hrs post infection. The remaining three plates were untreated and served as infected controls. One set of two plates, one with prodrug, one without, was harvested on day 7, 8, and 9 post infection.

These results corroborate the previous data and extend it. Increased cell death is seen at all time points in infected pro-drug treated cells relative to infected but

untreated cells. These data also reveal that the bystander effect is more pronounced as the infection becomes more advanced. When 5-FC is added at 24 hours and at 48 hours post infection, cell death is greater than when the prodrug is added immediately after initial infection. These data demonstrate that a tissue specific adenovirus harboring the cd gene has superior killing ability to wild type adenovirus.

6. Attenuated Ad5 with PSE Driving E1A and SV40 T Antigen in ΔE3 to Increase Host Range to Include Monkey and Human Cells

Human adenovirus does not efficiently replicate in monkey cells. Associated with decreased levels of fiber mRNA in the cytoplasm, the abortive infection is caused by defects in the late gene expression regulated by E4 proteins (Ross and Ziff (1992) *J. Virology* **66**:3110-3117). Adenovirus-SV40 hybrids -- shown to contain a small portion of the SV40 genome coding for the large T antigen integrated into the E3 region of the adenovirus 2 genome, overcome this defect and lyse monkey cells (Lewis and Rowe (1970) *ibid* **5**:413-420; Lewis et al, (1973) *ibid* **11**:655-664). The large T antigen (Tag) is believed to confer this host-range capability on these hybrids (Tijan et al., (1979) *PNAS* **75**:1279-1283). Several Ad2-SV40 hybrids have been isolated from SV40 and Ad2 infected cultures, each containing a conserved amount of the Tag carboxy terminal coding region and varying lengths of amino terminal coding region.

We have adopted this paradigm to develop Ad5 tissue specific, host-range mutants for use in monkey studies. Two strategies were undertaken. The first used the host-range mutant Ad2+ ND1, which harbors SV40 Tag coding sequence from map units 0.28-0.11, as a model (Zain & Roberts (1978) *J. Mol. Biol* **120**:13). A 666 base pair PstI/BamHI restriction fragment in the plasmid pDIS (obtained from Edgar Schrieber), a plasmid which contains the entire Tag coding sequence, the endogenous SV40 early promoter, and an inverted SV40 enhancer, contains the appropriate 3' sequence and was cloned via the shuttle plasmid pABS4 (Microbix) into the unique

PacI restriction site in the E3 region of BHG11 (Microbix). Upstream of the coding sequence was cloned an oligo (+) strand:

26.99.1, 5'-GTTTGTGTATTTTAGATCAAAGATGCTGCA (SEQ ID NO:38), and (-) strand:

26.99.2, 5'-GCATCTTTGATCTAAAATACACAAAC (SEQ ID NO:39), that contains a splicing acceptor sequence, ribosome recognition sequences, and an ATG to achieve expression of the appropriate peptide (CN170). Expression of this construct is dependent on a transcript originating from the major late promoter.

The second strategy involved creating an internal deletion in the Tag sequence in the plasmid pDIS between the EcoNI site in the amino terminal region and the PstI site in the carboxy terminal coding sequence by using an adapter oligo (+) strand:

27.183.1, 5'-TAAAGGAGGAGATCTGCCTAAAACACTGCA (SEQ ID NO: 40), and (-) strand:

27.183.2, 5'-GTGTTTTAGGCAGATCTCCTCCTTT (SEQ ID NO:41).

The entire transcription unit, including the enhancer, promoter, and the coding sequence was excised by HpaII/BamHI digestion and cloned via shuttle plasmid into the unique PacI site of BHG11 (CN183). This method generates a discrete transcription unit in Ad5 sequence whose expression is not dependent on the major late promoter.

Two host-range Ad5-SV40 viruses were produced. Both contain the carboxy termini of the Tag but lack the promoter. One is a tissue-specific, attenuated virus with the prostate specific enhancer (PSE) modulating expression of the EIA proteins (CN725). The other is wild type Ad5 with a Tag insertion (CN726). Both were generated by homologous recombination by cotransfecting 293 cells, a human kidney cell line that expresses Ad EIA and EIB proteins, with CN145(PSE-EIA) or pXCl (wild type Ad5 left hand end) and CN170.

Host-Range Mutant Characterization. Wild type Ad5 (CN702) and CN726 were plaqued on both 293 cells and CV1 cells, an African Green Monkey kidney cell

line. Plaques were counted in both cell monolayers and a ratio between the plaques in the two cell lines was determined. The ratio for CN726 and CN702 was 0.01 and 0.0007, respectively. The capability of replication of adenovirus in monkey cells allows preclinical evaluation of recombinant attenuated adenoviruses in monkeys, yielding valuable information for dosage and formulation of these viruses as therapeutic agents in humans.

7. Construction of Recombinant DNA to Introduce Mutations in E2, the DNA Binding Protein (DBP), for the generation of Recombinant Ad5 with Extended Host Range Allowing Replication in Human and Monkey Cells

Wild type adenovirus type 5 is only replication competent in human cells. For preclinical evaluation of therapeutic attenuated adenoviruses it would be desirable to test efficacy and toxicity in large human-like animals such as monkeys. A host range mutant hr404 has been described that confers a replication phenotype of human Ad5 in monkey cells (Klessig & Grodzicker (1979) *Cell* **17**:957-966). The nature of the hr404 mutation was shown to be a single point mutation C-- > T at adeno position 32657 in the DBP gene resulting in a change of Histidine to Tyrosine amino acid at codon 130 (Hl30Y) in the 72K DNA binding protein (Kruijer et al. (1981) *Nucleic Acids Res.* **9**:4439-4457).

We constructed a recombinant DNA molecule with the 5.8 kb EcoRI-BamHI fragment from plasmid BHG10 (Bett et al., *supra*) containing the right end sequences of Adenovirus type 5 and introduced by site-directed mutagenesis the H130Y mutation in the DBP gene. This plasmid should allow the construction of recombinant adenoviruses which are capable to replicate in human and monkey cells.

The 5769 bp EcoRI-BamHI fragment of BHG10 (Bett et al., *supra*) was cloned into similarly cut pBluescript KSII+ resulting in plasmid CN184. In order to eliminate disturbing restriction sites, a 2568 bp XhoI fragment was deleted yielding plasmid CN 186. The mutagenesis upper PCR primer reads:

28.180U, 5'-GCAACCCACCGGTGCTAATCAAGTATGGCAAAGGAGTAAGCGC-3 (SEQ ID NO:42)

The mutated T residue causing the H130Y mutation is shown in bold underlined style. Shown in italics is the unique SgrAI site in pCN186. The lower PCR primer reads:

28.180L, 5'-TGGCCTTGCTAGACTGCTCCTTCAGC-3' (SEQ ID NO:43)

PCR amplification was done with 100 pMol of each of these primers, 200 ng CN186 as template, 1 mM dNTP, lx Pfu buffer (Stratagene), dH2O to 100 µl, and 5U cloned Pfu polymerase (Stratagene) at 94°C 1 minute, 60°C 1 minute, 72°C 2 minutes for 30 cycles. The PCR yielded the expected DNA fragment of 588 bp. The DNA fragment was purified with a Wizard DNA clean-up column (Promega) and digested with restriction enzymes SgrAI and AflII. The 473 bp fragment of interest containing the H130Y mutation was gel purified and isolated. For reinsertion into the DBP gene, the mutated DNA fragment was ligated with the 1639 bp AscI-SgrAI fragment from CN184 and the 6609 bp AflII-AscI fragment from CN184 resulting in plasmid CN188.

Recombinant adenovirus genomes were constructed by *in vitro* ligation of the 5.8 kb EcoRI-BamHI fragment of CN188 with a 21562 bp EcoRI-Bst1107 center DNA fragment of BHG10 and Bst1107-cut plasmid CN144. The resultant virus was designated CN723.

The capability of replication of adenovirus in monkey cells allows preclinical evaluation of recombinant attenuated adenoviruses in monkeys, yielding valuable information for dosage and formulation of these viruses as therapeutic agents in humans. Further, with the use of the hr404 mutation in CN723, the same virus used for monkey studies can be used as the human clinical trial virus.

8. Deletion of ORF 1,2,3 and part of ORF 4 from the E4 Region of Adenovirus Type 5

The E4 region codes for two polypeptides which are responsible for stimulating the replication of viral genomic DNA and for stimulating late gene expression. The protein products of open reading frames (ORFS) 3 and 6 can both perform these functions, however the ORF 6 protein requires interaction with the EIB 55K protein for activity while the ORF 3 protein does not. To further restrict viral replication to prostate epithelial cells E4 orfs 1-3 can be deleted, making viral DNA replication and late gene synthesis dependent on E4 ORF 6 protein. By combining such a mutant with sequences in which the EIB region is regulated by the PSE, a virus can be obtained in which both the EIB function and E4 function are dependent on the PSE driving EIB.

A virus of this type was constructed by combining sequences from the plasmid d11006 which contains an E4 deletion of ORFS 1-3 (Bridge & Ketner, *J. Virol.* (1989) **63**:631-638) with BHG10, followed by co-transfection with CN144 to construct a recombinant virus. The plasmid pd11006 is cleaved with AvrII and AgeI to isolate sequences containing the mutated E4 region. This DNA segment is used to replace the homologous segment of CN108 cleaved with the same enzymes.

CN108 contains the 6 kb EcoRI fragment from BHG10 cloned into BSKSII+. Due to the E3 deletion in BHG10, the AvrII site at Ad5 nt 28752 had been deleted. AvrII still cut CN108 at Ad5 nt 35463; AgeI cut CN108 at Ad5 nt 31102. The 4.4 kb AvrII/AgeI fragment from CN108 was replaced with the 3.8 kb AvrII/AgeI fragment from d11006 producing CN203 containing the E4 deletion. The EcoRI fragment from CN203 was cloned into BHG10 to construct CN204. Homologous recombination of CN204 and CN144 yielded the virus CN726.

A similar virus of this type was constructed in the following manner. As previously described AvrII cut CN108 at Ad5 nt 35463. SapI cut CN108 twice, with one of the sites at Ad5 nt 34319. A complete AvrII cut and a partial SapI cut of

CN108 and religation removed 1144 bp from E4 yielded CN205. The 5.3 kb EcoRI/BamHI fragment from CN205 was cloned into similarly cut CN188 yielding CN206. The 14 kb BamHI fragment of CN206 containing both the E4 deletion and the hr404 mutation was cloned in BamHI cut BHG10 producing CN207. Homologous recombination of CN144 and CN207 in 293 cells yielded CN727.

9. PSE Controlling the E2 Region of Ad5

The E2 region of Adenovirus 5 codes for proteins related to replication of the adenoviral genome, including the 72 kDa DNA-binding protein, the 80 kDa precursor terminal protein and the viral DNA polymerase. The objective is to control expression of the E2 genes by the prostate-specific PSA enhancer/promoter in a recombinant adenovirus.

The E2 region of Ad5 is transcribed in a rightward orientation from two promoters, termed E2 early and E2 late, mapping at 76.0 and 72.0 map units, respectively. While the E2 late promoter is transiently active during late stages of infection and is independent of the EIA transactivator protein, the E2 early promoter is crucial during the early phases of viral replication.

The E2 early promoter, mapping in Ad5 from nt 27053-27121 consists of a major and a minor transcription initiation site, the latter accounting for about 5% of the E2 transcripts, two non-canonical TATA boxes, two E2F transcription factor binding sites and an ATF transcription factor binding site (for a detailed review of the E2 promoter architecture see Swaminathan & Thimmapaya, Current Topics in Microbiology and Immunology (1995) 199 part 3:177-194.

The E2 late promoter overlaps with the coding sequences of the L4 gene encoded by the counterstrand and is therefore not amenable for genetic manipulation. However, the E2 early promoter overlaps only for a few base pairs with sequences coding for a 33 k protein on the counterstrand. Notably, the SpeI restriction site (Ad5 position 27082) is part of the stop codon for the above mentioned 33 kDa protein and

conveniently separates the major E2 early transcription initiation site and TATA-binding protein site from the upstream transcription factor biding sites E2F and ATF. Therefore, an insertion of the PSA enhancer/promoter into the SpeI site would disrupt the endogenous E2 early promoter of Ad5 and should allow prostate-restricted expression of E2 transcripts.

Construction of recombinant Ad5 with the PSA enhancer/promoter in the E2 early promoter region. The BamHI-EcoRI fragment of Ad5 (positions 21562-27331) encompassing the E2 region was previously subcloned into pBluescript KSII+ resulting in plasmid CN184. A variant of this plasmid, CN188, carrying a mutation in the DBP gene (Hl30Y) allowing extended host range applications has been constructed and described above.

Plasmid CN188 was used for insertion of the PSA enhancer/promoter into the E2 region. The plasmid was linearized with SpeI and the 5' protruding ends were dephosphorylated with calf intestine alkaline phosphatase and then end-filled with Klenow polymerase and dNTP. The blunt ended PSE enhancer/promoter was ligated to SpeI linearized, blunt ended vector CN188. Recombinant DNAs with the PSE enhancer/promoter in the appropriate orientation for directing transcription initiation into the E2 region were identified. Plasmid CN196 contains the PSE enhancer/promoter in the backbone of CN188. The 5.3 kb EcoRI fragment of plasmid CN205, containing a deletion of the orf 1, 2, 3 and 4 of the E4 gene, was inserted in the appropriate orientation into EcoRI cut CN196, yielding plasmid CN197.

A recombinant viral genome with the PSE enhancer/promoter controlling expression of the EIA, EIB and the E2 early genes and the hr404 mutation H130Y in the DBP gene and deletion of open reading frames 1, 2, 3, and 4 of the E4 gene was obtained by *in vitro* ligation of the 9152 bp BamHI-Bst11071 fragment of CN144 with the 15802 bp Bst11071-BamHI fragment of BHG10 and the 12425 bp BamHI fragment of CN197.

Virus Preparation

Viruses were prepared as described previously (above). Table 1, below, lists the combinations of right end and left end Ad5 plasmids used to generate recombinant Ad5 with the desired features:

Table 1

Virus	Name	Left End Plasmid	Right End Plasmid
PSE-E1A	CN704-708	CN96	BHG10
PSE-E1A	CN718	CN145	BHG10
PSE-E1B	CN711	CN125	BHG111
PSE-E1A/E1B	CN716	CN144	BHG10
PSE-E1A/E1B	CN717	CN144	BHG10
PSE-E4		pXC.1	CN135-BHG10
ΔEnh/PSE-E1A	CN724		BHG10
PSE-E1A, ΔE3 CMV-SV40 T Ag	CN725	CN96	CN183
PSE-E1A/E1B, with HR404	CN723	CN144	CN188, CN108, BHG10
with ΔE3 CMV-CD PSE-E1A/E1B. ΔE4 (d11006)	CN726	CN144	CN207
PSE-E1A/E1B, hr404, ΔE4	CN727	CN144	CN207

Results:

Virus construction and genomic structure.

In the initial round of construction three replication competent, prostate-specific adenoviruses were produced. CN706 which contains the PSE driving the expression of the E1A gene, CN711 which contains the PSE driving the expression of the E1B gene, and CN716 which contains the PSE driving E1A expression and the PSE driving E1B expression. The viruses were generated by homologous recombination in 293 cells and cloned twice by plaque purification. The structure of the genomic DNA was analyzed by PCR and sequencing of the junctions between the

inserted sequences and the Ad genomic sequences. All viruses contained the desired structures (data not shown).

Virus growth *in vitro*.

The growth of the viruses *in vitro* was characterized by two assays: a burst size assay to measure the amount of infectious particles produced in one round of infection and plaque assays to assess the growth of the viruses in various types of cells.

For the burst size assays either LNCaP cells (a CaP cell line which produces PSA) or HBL100 cells (a non-malignant breast epithelial cell line) were infected with virus at a multiplicity of infection (MOI) of 1 (5 x 105 PFU per sample). At various time points samples were harvested and the amount of infectious virus present measured by plaque assays on 293 cells. Table 2 shows that CN706 produced 6.3 x 10^6 pfU from an input of 5 x 10^5 pfu in LNCaP cells after 48 hours. In HBL100 cells the increase from the same amount of input virus was to 2.0 x 10^6 pfu. CN706 then yielded 13 pfu per input infectious particle in LNCaP cells which was 3 fold greater than that produced in HBL100 cells over the same time period.

Burst size assays on CN711 also revealed preferential growth in LNCaP cells versus HBL100 cells (Table 2). In LNCaP cells 5 x 10^5 Y pfu input virus produced 4 x 10^7 pfu at 48 hours while in HBL100 cells 8 x 10^6 pfu were obtained at 48 hours. This represented a 40 fold increase in virus in LNCaP cells or a 5 fold greater yield than in HBL100 cells.

The differential in virus production for CN716 showed a wider disparity between the two cell lines. In LNCaP cells 1.7 x 10^7 pfu were obtained after 48 hours while in HBL100 cells 8 x 10^5 pfu were obtained at the same time point. Therefore in LNCaP cells 34 infectious particles were produced for each input particle at 48 hours while for HBL100 1.6 infectious particles was produced.

These results indicate that the expression of the early genes ElA and ElB can be controlled by the inserted PSE. To further characterize this regulation, production of CN706 virus was assayed by the burst assay in LNCaP cells in the presence or absence of the testosterone analog R1881. Since the PSE is highly active in the presence of androgens but essentially inactive in the absence of androgens, the production of early proteins controlled by the PSE and therefore the production of virus should be sensitive to androgen levels. As shown in Table 3 in the absence of R1881, 3 x 10^6 pfu were obtained at 48 hours for a three fold increase over input virus. In the presence of 1 nM or l0 nM R1881 two to three fold more pfu were obtained at 48 hours. In contrast, with wild type adenovirus assayed in parallel, no difference was evident in pfu obtained in the presence or absence of R1881.

Table 2. Burst Assays

	LNCaP	**HBL100**
CN706	6.3 x 10_6	2.0 x 10^6
CN711	4 x 10_7	8 x 10^6
CN716	1.7 x 10_7	8 x 10^5

Table 3. R1881 induction

	0 nM R1881	**1 nM R1881**	**10 nM R1881**
CN706	3 x 10_6	8 x 10_6	5 x 106

To further assess the growth selectivity of CN706, CN711, and CN716, the viruses were analyzed in plaque assays in which a single infectious viral particle

produces a visible plaque by multiple rounds of infection and replication. The results of a representative assay are shown in Table 4.

Table 4. Plaque assay

	Cell line				
	293	**LNCaP**	**HBL100**	**TSU**	**A549**
CN702	$2.3 \times 10_5$	$4.1 \times 10_5$	$4.3 \times 10_5$	$1.1 \times 10_6$	5.1×10^5
CN706	$2.3 \times 10_5$	$4.4 \times 10_4$	$1.7 \times 10_3$	$5.4 \times 10_4$	2.9×10^4
CN711	$2.3 \times 10_5$	$5.5 \times 10_5$	$2.7 \times 10_5$	$1.6 \times 10_5$	2.6×10^5
CN716	$2.3 \times 10_5$	$6.9 \times 10_5$	$2.7 \times 10_3$	$4.4 \times 10_3$	4.1 x 104

Virus stocks were diluted to equal pfu/ml, then used to infect monolayers of cells for 1 hour. The inoculum was then removed and the cells were overlayed with semisolid agar containing medium and incubated at 37°C for one week. Plaques in the monolayer were then counted and titers of infectious virus on the various cells were calculated. The data were normalized to the titer of CN702 on 293 cells.

The wild type virus CN702 showed approximately equal titers on each of the five cell lines. In contrast, each of the PSE modified viruses displayed a variable pattern of growth on the different cell types. CN706 grew to a 10 fold lower titer on LNCaP cells as on 293 cells, however, its titer on HBL100 cells was 260 fold lower than on 293 cells. On the non-PSA secreting CaP cell line TSU the titer of CN706 was approximately the same as on LNCaP cells which do secrete PSA. Similarly, the titer on the lung cell line A549 was also close to that on LNCaP cells. The virus CN711 displayed no significant difference in titer on the cell lines tested.

The data for the CN716 virus revealed a marked selectivity for growth in the LNCaP cell line. This virus grew well in LNCaP cells, reaching an even higher titer than on 293 cells. Growth of the virus on other cell lines was significantly lower, 18 fold lower on the next highest titer line, A549. The greatest differential was on

HBL100 cells, where the titer was 225 fold lower relative to that on LNCaP cells. The data from the burst size assay and the plaque assay demonstrate that human adenovirus can be modified using the PSE to develop viruses with selective growth properties for PSA secreting CaP cells.

EXAMPLE 2

Treatment of LNCaP tumor xenografts

The ultimate objective in the development of prostate-specific viruses is to treat patients with prostate disease. The feasibility of this objective was tested using LNCaP tumor xenografts grown subcutaneously in Balb/c nu/nu mice. The test viruses were inoculated into the mice either by direct intratumoral injection of approximately 10^8 pfu of virus in 0.1 ml PBS + 10 % glycerol or intravenously via the tail vein. Tumor sizes were measured and, in some experiments, blood samples were taken weekly.

The effect of intratumoral injection of CN706 on tumor size and serum PSA levels was compared to sham treatment. The sizes of the CN706 treated tumors continued to increase for two weeks, then progressively decreased for the duration of the experiment. At the end of the experiment all of the CN706 treated tumors (10 total) had diminished in size and five mice were cured of their tumor. In contrast, the buffer treated tumors continued to grow for the duration of the experiment, reaching approximately twice their original size by 42 days.

Previously published results have shown that serum PSA levels correlate with tumor size in the LNCaP tumor xenograft model. Measurement of PSA levels in the mice with tumors treated with CN706 indicated a rise in PSA levels one week after treatment, followed by a steady decline in PSA levels out to 35 days. Serum PSA

levels increased during the course of the experiment, averaging over 250 ng/ml at 35 days.

While it is likely that a therapeutic based on the viruses described here would be given intralesionally, it would also be desirable to determine if the virus can affect tumor growth following intravenous administration. If so, then it is conceivable that the virus could be used to treat metastatic tumor deposits inaccessible to direct injection. Groups of five mice bearing LNCaP tumors were inoculated with 10^8 pfu of CN706 by tail vein injection, or 10^8 pfu of a replication-defective adenovirus (CMV-LacZ) to control for non-specific toxic effects of the virus, or with buffer used to carry the virus. Tumors in mice treated with buffer or CMV-LacZ continued to grow for the duration of the experiment, ultimately reaching approximately five times their original size on average. Tumors in mice treated with CN706 grew slightly between the time of inoculation and the first measurement at 7 days, then the average tumor size diminished to approximately 75% of the original tumor volume by day 42.

Treatment of LNCaP tumors in nude mice with CN711 resulted in a similar outcome to treatment with CN706. In the CN711 treated animals (5 total) the tumors continued to grow between inoculation and day 8. Thereafter the average tumor size diminished, reaching 65% by day 49. The average tumor size of the buffer treated mice (4 total) increased through the duration of the experiment, reaching 300% of the original tumor volume by 49 days.

The same experimental protocol was used to test the CN716 virus in LNCaP tumors. Mice were inoculated with PBS+10% glycerol, CN716, or CN702. The tumors in the buffer mice grew rapidly and the mice were sacrificed due to large tumor sizes after three weeks. Tumors treated with CN702 continued to grow for two weeks, then diminished in size to 80% of their original volume by day 42. Tumors treated with CN716 remained at their original size for one week, then diminished in size to 40% of their original size by day 42. At the end of the experiment 2 of the 4 mice treated were cured of their tumors.

EXAMPLE 3

Construction of Replication-Competent Adenoviral Vectors in Which Adenoviral Genes are Under Transcriptional Control of Alpha-Fetoprotein TRE

A replication-competent adenoviral vector, CN733, was constructed in which multiple copies of the Alpha Fetoprotein Transcriptional Response Element (AFP-TRE) were placed upstream of adenovirus genes E1A and E1B, as shown schematically in Figure 1. AFP-TRE is depicted in SEQ ID NO:44. An alternative AFP-TRE is depicted in SEQ ID NO:45.

Cloning Strategy for Vector Construction

A human embryonic kidney cell line, 293, efficiently expresses E1A and E1B genes of Ad5 and exhibits a high transfection efficiency with adenovirus DNA. For these experiments, 293 cells were co-transfected with one left end Ad5 plasmid and one right end Ad5 plasmid. Homologous recombination generates adenoviruses with the required genetic elements for replication in 293 cells which provide E1A and E1B proteins *in trans* to complement defects in synthesis of these proteins.

The plasmids to be combined were co-transfected into 293 cells using cationic liposomes such as Lipofectin (DOTMA:DOPE™, Life Technologies) by combining the two plasmids, then mixing the plasmid DNA solution (10 μg of each plasmid in 500 μl of minimum essential medium (MEM) without serum or other additives) with a four-fold molar excess of liposomes in 200 μl of the same buffer. The DNA-lipid complexes were then placed on the cells and incubated at 37°C, 5% CO_2 for 16 hours. After incubation the medium was changed to MEM with 10% fetal bovine serum and the cells are further incubated at 37°C, 5% CO_2, for 10 days with two changes of medium. At the end of this time the cells and medium were transferred to tubes, freeze-thawed three times, and the lysate was used to infect 293 cells at the proper dilution to detect individual viruses as plaques.

Plaques obtained were plaque purified twice, and viruses were characterized for presence of desired sequences by PCR and occasionally by DNA sequencing. For further experimentation, the viruses were purified on a large scale by cesium chloride gradient centrifugation.

Using the above procedure, three replication competent, hepatocarcinoma cell-specific adenoviruses were produced: CN732, which contains an AFP-TRE driving the expression of the ElA gene; CN733, which contains two AFP-TREs driving expression of the E1A and ElB genes; and CN734, which contains an AFP-TRE driving E1B expression. The viruses were generated by homologous recombination in 293 cells and cloned twice by plaque purification. The structure of the genomic DNA was analyzed by PCR and sequencing of the junctions between the inserted sequences and the Ad genomic sequences to confirm that the viruses contained the desired structures. The structure of the viruses was also confirmed by Southern blot.

Table 5 lists the combinations of right end and left end Ad5 plasmids used to generate recombinant Ad5 with the desired features.

Table 5. Adenovirus vectors containing AFP-TRE

Virus	Name	Left End Plasmid	Right End Plasmid
E1A-AFP	CN732	CN219	BHG10
ElA/ElB-AFP	CN733	CN224	BHG10
E1B-AFP	CN734	CN234	BHG10

Adenoviral Vector Construction

Plasmid pXC.1 was purchased from Microbix Biosystems Inc. (Toronto). pXC.1 contains Ad5 sequences from (nucleotide) 22 to 5790. We introduced an AgeI site 12 bp 5' to the E1A initiation codon (Ad5 547) by oligo-directed mutagenesis and linked PCR. To achieve this, pXC.1 was PCR amplified using primers:

5'-TCGTCTTCAAGAATTCTCA (15.133A) (SEQ ID NO:14), containing an EcoRI site, and

5'-TTTCAGTCACCGGTGTCGGA (15.134B) (SEQ ID NO:15), containing an extra A to introduce an AgeI site. This created a segment from the EcoRI site in the pBR322 backbone to Ad5 560. A second segment of pXC.1 from Ad 541 to the XbaI site at Ad nucleotide 1339 was amplified using primers:

5'-GCATTCTCTAGACACAGGTG (15.133B) (SEQ ID NO:16) containing an XbaI site, and

5'-TCCGACACCGGTGACTGAAA (15.134A) (SEQ ID NO:17), containing an extra T to introduce an AgeI site. A mixture of these two PCR-amplified DNA segments was mixed and amplified with primers 15.133A and 15.133B to create a DNA segment from the EcoRI site to the XbaI site of pXC.1. This DNA segment encompasses the leftmost 1317 bases of Ad sequence and contains an AgeI site at Ad 547. This DNA segment was used to replace the corresponding segment of pXC.1 to create CN95.

An EagI site was created upstream of the E1B start site by inserting a G residue at Ad5 1682 by oligonucleotide directed mutagenesis as above. To simplify insertion of an AFP-TRE in the EagI site the endogenous EagI site in CN95 was removed by digestion with EagI, treatment with mung bean nuclease, and re–ligation to construct CN114. The primers:

5'-TCGTCTTCAAGAATTCTCA (15.133A) (SEQ ID NO:14), containing an EcoRI site, and

5'-GCCCACGGCCGCATTATATAC (9.4) (SEQ ID NO:46), containing an EagI site, and

5'-GTATATAATGCGGCCGTGGGC (9.3) (SEQ ID NO:47) containing an extra G and an EagI site, and

5'-CCAGAAAATCCAGCAGGTACC (24.020) (SEQ ID NO:30), containing a KpnI site, were used to amplify the segment between 1682 and the KpnI site at Ad5 2048. Co-amplification of the two segments with primers 15.133A and 24.020

yielded a fragment with an EagI site at Ad5 1682 which was used to replace the corresponding EcoRI/KpnI site in pXC.1 to construct CN124.

For construction of CN732, human AFP enhancer domains A and B (included in the region -3954 bp to -3335 bp relative to the AFP cap site) were PCR amplified from human genomic DNA (Clontec, Palo Alto, CA) using the following primers:

5' GTGACCGGTGCATTGCTGTGAACTCTGTA 3' (39.055B) (SEQ ID NO:48)

5' ATAAGTGGCCTGGATAAAGCTGAGTGG 3' (39.044D) (SEQ ID NO:49)

The AFP promoter was amplified from -163 to +34 using the following primers:

5' GTCACCGGTCTTTGTTATTGGCAGTGGT 3' (39.055J) (SEQ ID NO:50)

5' ATCCAGGCCACTTATGAGCTCTGTGTCCTT 3' (29.055M) (SEQ ID NO:51)

The enhancer and promoter segments were annealed, and a fusion construct was generated using overlap PCR with primers 39.055B and 39.055J. This minimal enhancer/promoter fragment was digested with PinA1 and ligated with CN124 using the engineered AgeI site 5' of the E1A cap site to produce CN219. The liver specific viral vector CN732 was generated by homologous recombination by cotransfecting 293 cells with CN219 and BHG10.

CN733 was constructed by using the following two PCR primers to amplify the enhancer/promoter element described above (-3954 to -3335 and -174 to +29):

5' TATCGGCCGGCATTGCTGTGAACTCT 3' (39.077A) (SEQ ID NO:52)

5' TTACGGCCGCTTTGTTATTGGCAGTG 3' (39.077C) (SEQ ID NO:53)

The PCR product was digested with EagI and ligated into similarly cut CN219. The resulting plasmid, CN224, contains two identical AFP regulatory elements, one each modulating expression of the E1A gene and the E1B gene.

CN733 was generated by homologous recombination in 293 cells by cotransfecting CN224 and BHG10.

To make CN734, the AFP-TRE regulating the expression of the E1A gene was excised from CN224 by digesting the plasmid with PinA1 and religating the vector. The resulting plasmid, CN234, was co-transfected with BHG10 in 293 cells to generate CN734.

Adenovirus Growth In Vitro

Growth selectivity of CN732, CN733, and CN734 was analyzed in plaque assays in which a single infectious particle produces a visible plaque by multiple rounds of infection and replication. Virus stocks were diluted to equal pfu/ml, then used to infect monolayers of cells for 1 hour. The inoculum was then removed and the cells were overlayed with semisolid agar containing medium and incubated at 37°C for 10 days (12 days for Table 8). Plaques in the monolayer were then counted and titers of infectious virus on the various cells were calculated. The data were normalized to the titer of CN702 (wild type) on 293 cells. The results of four representative assays are shown in Tables 6-9.

Table 6. Plaque assay for 733 (E1A/E1B)

Cell line	Virus	Titer	Avg. titre	Titre/293	702/733
293 (control)	733	2.70×10^6	2.65×10^6	1	N/A
	733	2.60×10^6			
	702	1.30×10^6	1.70×10^6	1	
	702	2.10×10^6			
Hep3B (AFP^+)	733	1.01×10^7	1.02×10^7	3.7	.37
	733	1.03×10^7			
	702	1.00×10^6	7.02×10^5	1.36	
	702	5.00×10^5			
HepG2 (AFP^+)	733	9.70×10^6	1.04×10^7	3.92	0.292
	733	1.10×10^7			
	702	1.60×10^6	1.95×10^6	1.14	
	702	2.30×10^6			
LNCaP (AFP^-)	733	4.00×10^3	3.00×10^3	0.0011	290
	733	2.00×10^3			
	702	4.00×10^5	5.05×10^5	0.32	
	702	7.00×10^5			
HBL100 (AFP^-)	733	0	0	0	100-1000
	733	0			
	702	1.00×10^2	3.07×10^2	0.00022	
	702	6.40×10^2			

Table 7. CN732, CN733, CN734 Plaque Assay Data

Cell line	Virus	Ave Titre	Titre/293	7XX/702
293 (control)	702	1.2×10^{6}	1	
	732	6.15×10^{5}	1	
	733	2.20×10^{6}	1	
	734	2.50×10^{5}	1	
Huh-7				
	702	1.10×10^{4}	0.01375	
	732	1.10×10^{5}	0.1788	13
	733	8.50×10^{4}	0.0386	3
	734	1.90×10^{4}	0.076	6
Sk-Hep-1				
	702	9.00×10^{2}	0.00113	
	732	0	0	0
	733	0	0	0
	734	1.00×10^{3}	0.004	4
HeLa				
	702	2.45×10^{2}	0.00030625	
	732	0	0	0
	733	1.5	6.81×10^{-7}	0.0022
	734	2.50×10^{3}	0.01	32
MCF-7				
	702	3.10×10^{3}	0.003875	
	732	7.5	1.22×10^{-5}	0.0031
	733	2.30×10^{1}	1.05×10^{-5}	0.0027
	734	1.70×10^{3}	0.0068	2
DLD-1				
	702	1.70×10^{3}	0.00213	
	732	1.40×10^{1}	2.28×10^{-5}	0.011
	733	1	4.54×10^{-7}	0.00021
	734	1.55×10^{3}	0.0062	3

Table 8. CN732, CN733, CN734 Plaquing Efficiency

Cell line	Virus	Titre
293	702	1×10^7
	732	1×10^7
	733	1×10^7
	734	1×10^7
HepG2 (AFP^+)	702	5×10^6
	732	3×10^6
	733	3×10^6
	734	1×10^7
Sk-Hep-1 (AFP^-)	702	6×10^4
	732	0
	733	0
	734	3×10^4
OVCAR-3 (AFP^-)	702	8×10^5
	732	0
	733	0
	734	3×10^4
HBL-100 (AFP^-)	702	2×10^6
	732	0
	733	0
	734	1×10^4

Table 9. Plaque assay for CN732, CN733, and CN734

Cell line	Virus	Ave Titre	Titre (cell line)/ Titer 293	CN7XX/CN702
293 (control)	702	5.0×10^6	1	
	732	4.8×10^6	1	
	733	3.2×10^6	1	
	734	3.0×10^8	1	
HepG2 (AFP^+)	702	2.3×10^7	4.6	-
	732	3.2×10^7	6.7	1.5
	733	6.0×10^6	1.9	0.41
	734	4.2×10^8	1.4	0.30
DU145 (AFP^-)	702	2.2×10^6	0.44	-
	732	3.0×10^4	0.0063	0.0143
	733	3.1×10^3	0.00097	0.002
	734	1.0×10^7	0.033	0.075
HBL-100 (AFP^-)	702	4.0×10^5	0.8	-
	732	0	-	0
	733	0	-	0
	734	6.0×10^6	0.02	0.025

OVCAR-3 (AFP$^-$)	702	3.3 X 10^5	0.066	-
	732	0	-	0
	733	0	-	0
	734	3.1 X 10^5	0.001	0.015

The wild type virus CN702 produced plaques on each of the cell lines tested. The number of plaques produced by CN702 was used as a base line against which to compare plaque formation by CN733.

In 293 cells growth of the viruses should be independent of the alterations to the E1 region due to the trans complimentation in this cell line. As expected, both CN702 and CN733 produced similar numbers of plaques on 293 cells.

Regarding the data from Table 6, in the AFP positive cell lines Hep3B and HepG2 CN702 produced similar numbers of plaques relative to 293 cells. In contrast, CN733 produced approximately four fold more plaques in the AFP positive cell lines than in 293 cells. The super normal level of plaque formation by CN733 in the AFP positive lines indicates that the AFP enhancer is active in these cells.

In the AFP negative cell lines LNCaP and HBL100 growth of both viruses was curtailed but to different extents. Wild type CN702 virus produced plaques in LNCaP cells at approximately 30% of the level seen in 293 cells. In HBL-100 cells CN702 formed plaques at 0.02% of the level formed in 293 cells. CN733 plaque formation was diminished even further in these AFP negative cell lines relative to CN702. In LNCaP cells CN733 produced plaques at a level 0.1% of that seen in 293 cells. In HBL100 cells CN733 did not produce plaques at all. In comparison to CN702, the growth of CN733 on AFP negative cell lines was reduced by at least 100 fold. This compares favorably with previous results where the E1B promoter of Ad40 was shown to specify a differential of approximately 100 fold between gut and conjunctival epithelial tissues (Bailey et al., 1994) and with deletion mutants of the E1B gene which were shown to specify a 100 fold differential in Ad growth between p53+ and p53- cells (Bischoff et al., 1996). Lastly, comparison of the titer of an

AFP+ cell type to the titer of an AFP- cell type provides a key indication that the overall replication preference is enhanced due to depressed replication in AFP- cells as well as the replication in AFP+ cells.

Regarding the data from Table 7, several observations can be made. First, CN732, CN733, and CN734 all plaque as efficiently in Huh-7 cells as CN702. In contrast, the plaquing efficiency for two of the adenoviruses (CN732 and CN733) decreases dramatically in the non-AFP producing cell lines included in the experiment. In the non AFP producing hepatocellular carcinoma cell line Sk-Hep-1, CN732 and CN733 produced no plaques at the dilutions tested. The results are similar for these two viruses in HeLa, MCF-7, and DLD-1. CN702's efficiency in DLD-1 cells exceeds CN733's by over 4000 fold.

With respect to the data in Table 8 (in which titers are normalized to 1 X 10^7 in 293 cells), CN732, CN733, and CN734 plaqued similarly to wild type (CN702) in HepG2 cells. However, these viruses plaqued poorly compared to CN702 in cell lines that do not express AFP. CN732 and CN733 produced no plaques at the dilutions tested in SK-Hep-1, OVCAR-3 and HBL-100, thus displaying significant titer differential. This corresponds to at least a 10,000 fold difference with wild type in HBL-100 and OVCAR-3 and a 1,000 fold difference in SK-Hep-1. CN734 also plaqued less efficiently than CN702 in OVCAR-3 (25 fold) and HBL-100 (200 fold) cells.

The data of Table 9 suggest that CN732, CN733, and CN734 plaque as efficiently as CN702 in cells that express AFP. However, they do not plaque as efficiently as CN702 in cell lines that do not express AFP. For example, neither CN732 nor CN733 produced any plaques at the dilutions tested in HBL100 cells or OVCAR-3 cells. CN734's plaquing differential was not as striking as CN732's or CN733's in the cell lines tested. It plaqued 13-fold, 40-fold, and 67-fold less efficiently than wild type in DU145, HBL100, and OVCAR-3, respectively.

The plaque assay data demonstrate that human adenovirus can be modified using an AFP-TRE to develop viruses with selective growth properties for AFP producing cells, particularly AFP-producing tumor cells such as hepatic carcinoma cells.

Western Analysis of E1A Expression from CN733

In the next experiment, we examined the effect of an AFP-TRE on the accumulation of E1A protein in CN733 infected cells. We reasoned that if one of the AFP regulatory regions installed in CN733 was modulating the E1A gene, the level of E1A protein in infected cells should also be affected. A western blot was conducted to test our hypothesis.

CN733's E1A accumulation was evaluated in Huh-7, SK-Hep-1 and DLD-1 cells. Monolayers were infected with either CN702 or CN733 at an MOI of ten and the harvested at various time points after infection. Samples were electrophoresed through a 10% acrylimide gel and transferred by electrophoresis to a nitrocellulose membrane. E1A protein was detected by using the ECL Western Detection system (Amersham, Arlington Heights, IL) using the suggested protocol. The primary antibody used was rabbit anti-Ad2 E1A antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The results are shown in Figure 2(A).

E1A accumulated rapidly in CN702 and CN733 infected Huh-7 cells. A high level of E1A was also detected in CN702 infected Dld-1 cells. However, little E1A protein was detected in CN733 infected Dld-1 cells. This result is intriguing because it suggests that CN733's poor plaquing efficiency in non AFP producing cell lines could be attributed to its restricted E1A expression. These data are consistent with the hypothesis that the AFP-TRE affects CN733's compromised replication in non-permissive cell types.

The experiment was repeated using Sk-Hep-1 cells as non AFP producing cells. Data were obtained after 24 hours post-infection. The results are shown in

Figure 2(B). The conclusion of this experiment is the same as the previous experiment: E1A expression is tightly regulated by the AFP-TRE.

Growth of CN733

CN733's growth in AFP- and non-AFP-producing cells was evaluated. Monolayers of Huh-7, Sk-Hep-1, and Dld-1 cells were infected at an MOI of ten with either CN702 or CN733. At various times after infection, duplicate samples were harvested, freeze-thawed three times, and titered on 293 cells to determine the total virus yield. Virus yield curves for CN702 and CN733 are plotted in Figures 3(A)-(C).

CN702 and CN733 grew efficiently in Huh-7 cells. Huh-7 cells produced similar amounts of infectious CN702 and CN733. In contrast, CN733's growth was severely restricted in SK-Hep-1 cells. CN702's titer at the conclusion of the experiment is about 1000 times greater than CN733's titer. The results were similar in Dld-1 cells.

The growth experiment was also performed to compare growth of CN732, CN733, and CN734 in HepG2 cells. Monolayers of HepG2 cells were infected at a multiplicity of infection (MOI) of two and harvested at various times after infection. Samples were titered on 293 cells to determine the final virus yield. The results are shown in in Figures 4(A)-(C). The data demonstrate that the adenovirus containing AFP-TREs grow efficiently in this cancer cell line. CN732, CN733, and CN734 each reach a high final titer at 36 hours post infection that is similar to that of CN702.

In another experiment, propagation was evaluated in primary hepatocytes (hNheps) isolated from a donor (32 year old black male) three days before the start of the experiment. Monolayers of cells were infected with virus at an MOI of two, harvested at various times after infection and titered on 293 monolayers. The results are shown in Figures 5(A) - (C). The data suggest that CN732 and CN733 grow less efficiently in hNheps than CN702. CN732's growth is delayed by twenty-four hours compared to CN702's. At thirty-six hours post infection, there is over ten fold more

infectious CN702 than CN733. CN733's growth is delayed by thirty-six hours. At thirty-six hours post infection, there is nearly 1000 times more infections CN702 than CN733. CN734 grows similarly to CN702. The data also suggest that CN733 has the most restrictive phenotype, followed by CN732 and CN734. Taken together, these results also indicate that an AFP-TRE inserted upstream of the E1A gene may be more effective in restricting host-range than an AFP-TRE engineered upstream of the E1B region. The presence of two AFP-TREs is even more effective.

In conclusion, the experiments described above indicate that it is possible to restrict an adenoviral vector's host range to AFP producing cells. As demonstrated by plaque assay and growth assay, the adenovirus vectors containing an AFP-TRE propagate efficiently in HepG2 and Huh-7 cells but poorly in non AFP producing cells.

Testing Cytotoxic Ability of Adenovirus Vector CN733 on HepG2 Tumor Xenographs

An HCC mouse xenograft model was used to evaluate CN733's potential as a therapeutic adenovirus for liver cancer. The AFP producing HCC cell line HepG2 was injected subcutaneously on the right flanks of Balb/c nu/nu mice. After allowing several weeks for the tumors to take, each was treated with an intratumoral injection of either 1.5×10^{11} particles of CN733 in PBS, glycerol or buffer alone. Eleven mice bearing HepG2 tumors were treated, six with CN733 and five with buffer. Tumors were measured weekly until the conclusion of the experiment. Tumor volume was calculated by multiplying the tumor's length by the square of its width and dividing the product by two. Figure 6(A) is a graph of average tumor volume for each treatment group vs. time.

In six weeks, HepG2 tumors challenged with buffer grew to over five times their original size. In contrast, tumor growth in CN733 treated mice was attenuated. One tumor even regressed to 3% of its maximum volume. These data suggest that CN733 invaded the tumors and delivered cytotoxicity.

In addition to monitoring tumor growth, we harvested serum samples and assayed AFP levels. The results are shown in Figure 7. The data suggest that serum AFP levels rises more slowly in mice receiving CN733 than in control mice receiving buffer.

In another experiment, antitumor activity of different administrative regimens was compared for CN733. Animals were treated with a single intramuoral administration of either buffer (n=8, volume=919 mm^3) or 1.5 X 10^{11} particles of CN733 (n=8, volume = 944 mm^3). A third group of animals was treated with five consecutive daily doses of 1.5 X 10^{11} particles of CN733 (n=8, volume=867 $mm^{3)}$. Despite the large systemic virus burden, the mice displayed no obvious signs of toxicity. Tumors were measured weekly by external caliper for four weeks after injection. Animals from groups treated with a single dose of CN733 and buffer were sacrificed four weeks after treatment because of excessive tumor burden. All animals from the group treated with five doses of CN733 survived until the conclusion of the study. Despite the large systemic virus burden, these animals showed no obvious signs of treatment related toxicity. The results are shown in Figure 6(B). On average, buffer treated tumors increased to three times their initial volume by four weeks after treatment. Tumors treated with a single dose of CN733 increased to nearly four times their initial volume. In contrast, tumors treated with five doses of CN733 remained the same volume. Five out of eight tumors (63%) responded to treatment. One animal had no palpable tumor at the end of the study.

Statistical analysis using the Students T-test suggests that there was no significant difference at any time point between buffer treated animals and those treated with one dose of CN733 ($p>0.5$). However, there was a significant difference between buffer treated animals and those treated with five doses of CN733 beginning at two weeks post injection ($p= 0.045$) and continuing through four weeks ($p= 0.034$).

The data suggest that CN733 exhibits significant antitumor activity in HepG2 nude mouse xenografts. CN733 administered daily for five consecutive days at a

dose of 1.5x 10^{11} particles can cause tumor regression in some animals. A single dose, however, is not sufficient to cause tumor killing.

In the first experiment, the tumors responded to a single dose of CN733 but did not appear to respond in the second. The inventors note that there is often a variation in tumor phenotype (including growth characteristics and AFP expression) from experiment to experiment.

In conclusion, the *in vivo* experiments suggest that CN733 causes significant tumor killing in large hepatoma xenografts. Five doses of intratumorally adminstered virus induced regression in four out of eight animals and cured one animal twenty-eight days after injection. On average, buffer treated tumors tripled while CN733 treated tumors remained the same.

EXAMPLE 4

Construction of Replication-Competent Adenoviral Vectors in Which Adenviral Genes are Under Transcriptional Control of Carcinoembryonic Antigen (CEA) TRE

Using the procedure described above in Example 3, three replication competent, CEA cell-specific adenoviruses were produced: CN741, which contains an CEA-TRE driving the expression of the EIA gene; CN742, which contains two CEA-TREs driving expression of the E1A and EIB genes; and CN743, which contains an CEA-TRE driving E1B expression. These constructs are shown schematically in Figure 8. The viruses were generated by homologous recombination in 293 cells and cloned twice by plaque purification. The structure of the genomic DNA was analyzed by PCR and sequencing of the junctions between the inserted sequences and the Ad genomic sequences to confirm that the viruses contained the desired structures.

Table 10 lists the combinations of right end and left end Ad5 plasmids used to generate recombinant Ad5 with the desired features.

Table 10. Adenovirus vectors containing CEA-TRE

Virus	Name	Left End Plasmid	Right End Plasmid
E1A-CEA	CN741	CN266	BHG11
E1A/E1B-CEA	CN742	CN285	BHG11
E1B-CEA	CN743	CN290	BHG11

A replication-competent adenoviral vector, CN742, was constructed in which copies of the Carcinoembryonic Antigen Transcriptional Response Element (CEA-TRE) were placed upstream of adenoviral genes E1A and E1B.

The Carcinoembryonic Antigen Transcriptional Response Element (CEA-TRE)

The transcriptional response element of the carcinoembryonic antigen (CEA-TRE), about -402 to about +69 bp relative to the transcriptional start (SEQ ID NO:54), was amplified by polymerase chain reaction (PCR) from human genomic DNA using primers:

5' ATT ACC GGT AGC CAC CAC CCA GTG AG 3' (39.174B, upper primer) (SEQ ID NO:55)

and

5' TAG ACC GGT GCT TGA GTT CCA GGA AC 3' (39.174D) (SEQ ID NO:56).

A unique restriction site, AgeI, was introduced by the primer pair at the ends of the PCR-amplified product.

The CEA-TRE PCR fragment was ligated into pGEM-T vector (Promega) which had been linearized with EcoRV. The ligation mixture was transformed into *E. coli* DH5α cells. The desired clone, carrying a CEA-TRE fragment, was obtained and designated CN265.

Construction of CEA-TRE Adenoviruses Comprising One or Two Adenovirus Genes Under Transcriptional Control of CEA-TRE

Three replication-competent, CEA cell-specific adenoviruses were produced:

CN741, which contains a CEA-TRE driving the expression of the E1A gene;

CN742, which contains two CEA-TREs driving expression of both the E1A and E1B genes; and

CN743, which contains a CEA-TRE driving E1B expression.

The viruses were generated by homologous recombination in 293 cells and cloned by plaque purification. The structure of the genomic DNA was analyzed by PCR and sequencing of the junctions between the inserted sequences and the Ad genomic sequences to confirm that the viruses contained the desired structures.

CEA-TRE-Driven E1A Adenovirus Plasmid (CN741)

Briefly, a CEA-TRE fragment was inserted into CN124 (a left-hand adenovirus plasmid, described below) to generate CN266, which comprises the left-hand end of adenovirus with a CEA-TRE controlling expressing of the adenovirus E1A gene. CN266 was recombined with a plasmid carrying the right-hand portion of adenovirus to generate CN741, which is a full-length adenovirus in which CEA-TRE controls expression of adenovirus gene E1A.

In more detail, the CEA-TRE sequence was excised from CN265 (described in Example 1) by digestion with PinAI.

CN124 is a derivative of construct pXC.1, which contains the wild-type (wt) left-hand end of Ad5, from nt (nucleotide) 22 to 5790, including both E1A and E1B [McKinnon (1982) *Gene* 19:33-42]. Plasmid pXC.1 was purchased from Microbix Biosystems Inc. (Toronto). An AgeI site was introduced 12 bp 5' to the E1A initiation codon (Ad5 nt 547) by oligo-directed mutagenesis and linked PCR. To achieve this, pXC.1 was PCR-amplified using primers:

15.133A, 5'-TCGTCTTCAAGAATTCTCA (SEQ ID NO:14), containing an EcoRI site, and

15.134B, 5'-TTTCAGTCACCGGTGTCGGA (SEQ ID NO:15), containing an extra A to introduce an AgeI site.

This created a segment from the EcoRI site in the pBR322 backbone to Ad5 nt 560.

A second segment of pXC.1 from Ad nt 541 to the XbaI site at Ad nucleotide 1339 was amplified using primers:

15.133B, 5'-GCATTCTCTAGACACAGGTG (SEQ ID NO:16) containing an XbaI site, and

15.134A, 5'-TCCGACACCGGTGACTGAAA (SEQ ID NO:17), containing an extra T to introduce an AgeI site.

These two PCR-amplified DNA segments were mixed and amplified with primers 15.133A and 15.133B to create a DNA segment from the EcoRI site to the XbaI site of pXC.1. This DNA segment encompasses the leftmost 1317 bases of Ad sequence and contains an AgeI site at Ad nt 547. This DNA segment was used to replace the corresponding segment of pXC.1 to create CN95.

An EagI site was created upstream of the E1B start site by inserting a G residue at Ad5 nt 1682 by oligonucleotide directed mutagenesis as above. To simplify insertion of a CEA-TRE in the EagI site, the endogenous EagI site in CN95 was removed by digestion with EagI, treatment with mung bean nuclease, and re-ligation to construct CN114. The following primers were used to amplify the segment between 1682 and the KpnI site at Ad5 nt 2048:

15.133A, 5'-TCGTCTTCAAGAATTCTCA (SEQ ID NO:14), containing an EcoRI site, and

9.4, 5'-GCCCACGGCCGCATTATATAC (SEQ ID NO:46), containing an EagI site

9.3, 5'-GTATATAATGCGGCCGTGGGC (SEQ ID NO:47), containing an extra G as well as an EagI site, and

24.020, 5'-CCAGAAAATCCAGCAGGTACC (SEQ ID NO:30), containing a KpnI site.

Co-amplification of the two segments with primers 15.133A and 24.020 yielded a fragment with an EagI site at Ad5 nt 1682, which was used to replace the corresponding EcoRI/KpnI site in pXC.1 to construct CN124.

A CEA-TRE fragment excised from CN265 (see above) by digestion with PinAI was ligated into similarly digested CN124 (which contains the left hand end of the adenovirus) to generate CN266. CN266 is a vector comprising the left-hand portion of adenovirus, in which a CEA-TRE is inserted upstream of and controls expression of E1A.

The full-length CEA-E1A virus, designated CN741, was constructed by homologous recombination of CN266 and BHG11, which contains the right hand side of Adenovirus 5. Briefly, the plasmid CN266 was digested with PvuI; BHG11, with ClaI. Equivalent amounts (5 μg) of each linearly cut plasmid were transfected into 293 cells with a 4-fold excess of cationic liposomes such as Lipofectin DOTAP/DOPE (1:1). 293 is a human embryonic kidney cell line which efficiently expresses the E1A and E1B genes of Ad5 and exhibits a high transfection efficiency with adenovirus DNA. 8 days after infection, viral plaques were observed on the cell monolayer; cells/viruses were harvested, freeze-thawed 3x, centrifuged to pellet the cellular debris, and the supernatant collected. CN741, the full-length adenovirus in which a CEA-TRE controls E1A expression, was plaque-purified three times.

In an alternative protocol for transfection of right- and left-hand adenovirus plasmids into 293 cells, the plasmids are first combined, then the plasmid DNA solution (10 μg of each plasmid in 200 μl of minimum essential medium without serum or other additives) is mixed with an 4-molar excess of liposomes (e.g., DOTAP/DOPE) in 200 μl of the same buffer. The DNA-lipid complexes are then placed on the cells and incubated at 37°C, 5% CO_2 for 16 hours. After incubation, the medium is changed to MEM with 10% fetal bovine serum and the cells are further incubated at 37°C, 5% CO_2, for two weeks with two changes of medium. At the end of this time the cells and medium are transferred to tubes, freeze-thawed three times, and the lysate is used to infect 293 cells at the proper dilution to detect individual viruses as plaques. Plaques obtained were plaque-purified twice, and viruses were characterized for presence of desired sequences by PCR and occasionally by DNA sequencing. For further experimentation the viruses are prepared on a larger scale by cesium chloride gradient centrifugation.

Several clones of CN741, the full-length adenovirus in which a CEA-TRE controls E1A expression, were characterized by PCR, Southern Blot, and the plaque assay for specificity.

1. **PCR**: Primers were used to amplify the region of clones of CN741 starting upstream of the CEA insert in the E1A region (primer 39.141C: 5' ATT TGT CTA GGG CCG GGA CTT 3' (SEQ ID NO:57)) and downstream at the 3' end of the ElB region (primer 39.141H : 5' CGC GCG CAA AAC CCC TAA ATA AAG 3' (SEQ ID NO:58)) of adenovirus. The amplified fragment is 4249 bp. The following clones tested positive by PCR: 46.130.7.4., 46.130.8.3, 46.130.9.1.1, 46.130.9.2.1, 46.130.9.3.1, and 46.130.9.4.1.

2. **Southern blot**: Positive clones of CN741 were further characterized by Southern blot. Viral DNA of CN741 clones was digested by the following enzymes: ScaI, AflII, and AflII/XbaI. The viral DNA was probed with a randomly primed fragment of E1A. The correct fragments were as follows: ScaI digest, 926 and 5645 bp; AflII digest, 4011 bp; and AflII/XbaI digest, 1817 bp. Each positive clone displayed the correct fragment pattern.

3. **Plaque assay**: The plaque assay is described in Example 2.

These assays confirmed the identity of CN741, the full-length adenovirus in which a CEA-TRE controls E1A expression.

CEA-TRE-Driven E1B Adenovirus Plasmid (CN743)

Briefly, a CEA-TRE fragment was inserted into CN124 (a left-hand Ad vector, described above) to generate CN290, which comprises the left-hand end of adenovirus with a CEA-TRE controlling expressing of the adenovirus E1B gene. CN290 was recombined with a plasmid carrying the right-hand portion of adenovirus to generate CN743, which is a full-length adenovirus in which CEA-TRE controls expression of adenovirus gene E1B.

In more detail, the CEA-TRE was obtained as an EagI fragment from CN284 (described below). This fragment was isolated by gel electrophoresis and inserted into CN124, similarly cut with EagI. CN124, also described above, contains the left-hand portion of Adenovirus 5, with an artificial EagI site upstream of the E1B start site. The resulting clone, designated CN290, has a CEA-TRE inserted upstream of the E1B in a left-hand portion of adenovirus. The identity of CN290 was confirmed by restriction digest (ScaI: 2937 and 7406 bp; SmaI: 180, 783, 2628, and 6752 bp).

CN743 was generated by homologous recombination by co-transfecting 293 cells, which produces E1B, with CN290 and BHG11, which contains the wt right hand portion of Ad5. Thus, CN743 is a full-length adenoviral genome in which gene E1B is under control of a CEA-TRE.

Construction of Adenovirus Vectors in Which Expression of Two Adenovirus Genes Are Each Controlled by a CEA-TRE (CN742)

Briefly, a CEA-TRE fragment was inserted upstream of the E1B gene in construct CN266, which already had a CEA-TRE fragment inserted upstream of E1A. The resulting plasmid was designated CN285 and contained a left-hand portion of adenovirus with separate copies of a CEA-TRE driving expression of E1A and E1B. CN285 was recombined with a right-hand portion of adenovirus to generate CN742, which is a full-length adenovirus in which expression of both E1A and E1B is controlled by CEA-TRE.

In more detail, CN285 was constructed by amplifying the CEA-TRE inserted into the E1A region (e.g., CN266) by PCR using primers:

5' TAA CGG CCG AGC CAC CAC CCA 3' (39.180A, upper primer) (SEQ ID NO:59) and

5' TAT CGG CCG GCT TGA GTT CCA GG 3' (39.180B, lower primer) (SEQ ID NO:60). The unique restriction site EagI was introduced by the primer pair

at the ends of the PCR-amplified product. The PCR product was ligated into pGEM-T Vector (Promega), and the resultant plasmid designated CN284.

The EagI CEA-TRE fragment was excised from CN284 and isolated by gel electrophoresis. The CEA-TRE fragment was ligated into CN266 which had been cut with EagI. CN266 (described above) is a left-hand portion of adenovirus in which a CEA-TRE controls expression of E1A. The resulting clone was confirmed by restriction digest (ScaI: 1682, 1732, and 7406 bp; SmaI: 783, 899 2628, and 6330 bp). The clone was designated CN285, which represents a left-hand portion of adenovirus in which both E1A and E1B are under control of separate CEA-TREs.

CN742 was generated by homologous recombination by co-transfecting 293 cells with CN285 and BHG11, which has the wt right hand portion of adenovirus. Thus, construct CN742 is a full-length adenoviral genome with genes E1A and E1B both under control of a CEA-TRE.

In short, full-length adenoviruses were constructed in which one or two adenoviral early genes were under transcriptional control of a CEA-TRE.

Comparative testing of virus growth in vitro

Growth selectivity of CN741, CN742 and/or CN743 (full-length adenoviruses in which one or two early genes is under control of a CEA-TRE) is analyzed in plaque assays in which a single infectious particle produces a visible plaque by multiple rounds of infection and replication. Virus stocks are diluted to equal pfu/ml, then used to infect monolayers of cells for 1 hour. Comparison of normalized titres in cells that allow a CEA-TRE to function and cells that do not allow a CEA-TRE to function indicates replication preference. Cells chosen for this study are cells that allow a CEA-TRE to function, such as NCIH508, LoVo, SW1463, MKN1, MKN28, MKN45 and cells that do not allow such function, such as HuH7 or HeLa. The inoculum is then removed and the cells are overlayed with semisolid agar containing medium and incubated at 37°C for one week. Plaques in the monolayer are then

counted and titers of infectious virus on the various cells are calculated. The data are normalized to the titer of CN702 (wild type) on 293 cells.

Full-length adenovirus CN741, in which transcription of E1A is under control of CEA-TRE, was tested in this way. Clone 46.130.8.3 was used, and CN702 (wt adenovirus) was a control. Plaques observed on cell lines were normalized to infectivity on control 293 Cells. The ratio of normalized plaques of CN741 and CN702 were compared to evaluate plaque preference in cell types. Table 2 depicts the plaque assay results. Cells examined were 293 (CEA-deficient), LoVo (CEA-producing), OVCAR (CEA-deficient), HBL100 (CEA-deficient), and HepG2 (CEA-producing). We have found that OVCAR and HBL100 cells do not express levels of CEA detectable by ELISA, using a standard protocol with a kit purchased from Genzyme. However, while we also found that HepG2 cell do not produce CEA detectable in the ELISA test, Zhai et al. [(1990) *Gastroenter.* 98:470-7] showed that HepG2 cells *do* produce CEA, as detectable by the PAP and avidin-biotin technique.

Table 11

Plaque assay results of CN741 (CEA-E1A) on human cell lines

Cell Line	**Normalized Plaques CN702 (wt)**	**Normalized Plaques CN741 (CEA-E1A)**	**Ratio of CN741/CN702**
293	1.0	1.0	1.0
LoVo	1.5	0.579	0.39
OVCAR	1.2	0.372	0.31
HBL100	0.75	0.085	0.11
HepG2	1.75	0.69	0.39

The plaque assay results in Table 11 indicate that the growth pattern of CN741 has been altered by the introduction of a CEA-TRE. In each cell line, the growth of the CN741 virus is reduced in comparison to wild-type adenovirus CN702. The ratio of CN741/CN702 in the CEA-proficient cell lines LoVo and HepG2 were similar. Importantly, there was a 4-fold reduction in the ability of CN741 to replicate in the CEA-deficient cell line HBL100 cells. These data seem to indicate that CN741 has a greater ability (i.e., more specificity for replication) in CEA-proficient cells (LoVo and HepG2) than in CEA-deficient cells (HBL100).

Curiously, the CN741/CN702 ratio was similar in OVCAR (CEA-deficient) to that in CEA-producing cells. This suggests that replication of the CEA-E1A adenovirus relative to wt virus in OVCAR (CEA-deficient) was similar to that in CEA-producing cells. There are several possible explanations for this finding. Note that HepG2, as stated above, was determined to be CEA-deficient a CEA ELISA assay, but revealed to be CEA-proficient by the PAP and avidin-biotin technique. The ELISA method may be similarly insufficient to detect low levels of CEA present in OVCAR. Alternatively, it is possible that OVCAR cells also produce CEA, but the protein is expressed too transiently or too quickly degraded to be detectable by ELISA, yet is somehow able to allow activation of transcription of a CEA-TRE and replication of CN741.

EXAMPLE 5

Construction of Replication-Competent Adenoviral Vectors in Which Adenoviral Genes are Under Transcriptional Control of Mucin TRE

Adenoviral vectors in which a MUC1-TRE controls expression of E1A and/or E1B were constructed. A construct (CN226) was built in which MUC1-TRE controls

E1A. In another vector, a MUC1-TRE was inserted upstream of the E1B gene in CN226. A third vector CN237 was constructed, in which MUC1-TRE mediates E1A and E1B expression.

Construction of CN226 (MUC1-TRE E1A)

Briefly, CN226, in which MUC1-TRE controls E1A expression, was constructed as follows. The MUC1-TRE region of SEQ ID NO:61 was amplified from human genomic DNA (Clonetech) by PCR (Perkin Elmer 2400) with the following primer pairs: 5' TAA TCC GGA CGG TGA CCA CTA GAG GG 3' (39.088A, upper primer-SEQ ID NO:62) and 5' TAT TCC GGA TCA CTT AGG CAG CGC TG 3' (39.088B, lower primer-SEQ ID NO:63). The primers were constructed with BspEI ends, which are compatible with the AgeI site in CN124. CN124 is a derivative of construct pXC.1, which contains the wild-type left-hand portion of Adenovirus 5 (Ad5), from nt 22 to 5790, including both E1A and E1B (McKinnon (1982) *Gene* 19:33-42). CN124 also has, among other alterations, an artificial AgeI site at Ad5 nt 547 (just upstream of the E1A transcriptional start at nt 498 and the E1A coding segment beginning with ATG at 610). CN124 also contains an artificial EagI site at Ad5 nt 1682, or just upstream of the E1B coding segment.

To construct CN124 from pXC.1, we introduced an AgeI site 12 bp 5' to the E1A initiation codon (Ad5 547) by oligonucleotide-directed mutagenesis and linked PCR. To achieve this, pXC.1 was PCR amplified using primers:

5'-TCGTCTTCAAGAATTCTCA (15.133A) (SEQ ID NO:14), containing an EcoRI site, and

5'-TTTCAGTCACCGGTGTCGGA (15.134B) (SEQ ID NO:15), containing an extra A to introduce an AgeI site. This created a segment from the EcoRI site in the pBR322 backbone to Ad5 560. A second segment of pXC.1 from Ad 541 to the XbaI site at Ad nucleotide 1339 was amplified using primers:

5'-GCATTCTCTAGACACAGGTG (15.133B) (SEQ ID NO:16) containing an XbaI site, and

5'-TCCGACACCGGTGACTGAAA (15.134A) (SEQ ID NO:17), containing an extra T to introduce an AgeI site. A mixture of these two PCR-amplified DNA segments was mixed and amplified with primers 15.133A and 15.133B to create a DNA segment from the EcoRI site to the XbaI site of pXC.1. This DNA segment encompasses the leftmost 1317 bases of Ad sequence and contains an AgeI site at Ad 547. This DNA segment was used to replace the corresponding segment of pXC.1 to create CN95.

An EagI site was created upstream of the E1B start site by inserting a G residue at Ad5 1682 by oligonucleotide directed mutagenesis as above. To simplify insertion of an AFP-TRE in the EagI site the endogenous EagI site in CN95 was removed by digestion with EagI, treatment with mung bean nuclease, and re–ligation to construct CN114. The primers:

5'-TCGTCTTCAAGAATTCTCA (15.133A) (SEQ ID NO:14), containing an EcoRI site, and

5'-GCCCACGGCCGCATTATATAC (9.4) (SEQ ID NO:46), containing an EagI site, and

5'-GTATATAATGCGGCCGTGGGC (9.3) (SEQ ID NO:47) containing an extra G and an EagI site, and

5'-CCAGAAAATCCAGCAGGTACC (24.020) (SEQ ID NO:30), containing a KpnI site, were used to amplify the segment between 1682 and the KpnI site at Ad5 2048. Co-amplification of the two segments with primers 15.133A and 24.020 yielded a fragment with an EagI site at Ad5 1682 which was used to replace the corresponding EcoRI/KpnI site in pXC.1 to construct CN124.

Amplification of the MUC1-TRE utilized an annealing temperature of 55°C (30 cycles) with an extension temperature and time of 72°C for 60 seconds. PCR products were purified with the QIAQuick Spin PCR Purification Kit (Qiagen). The MUC1-TRE PCR product was digested with BspI and ligated in front of the E1A region of CN124, which had been linearized with PinAI to AgeI ends. Ligation of

the insert into the vector destroyed the AgeI restriction site. The resulting clone was confirmed by restriction digest : HindIII/PinAI, 1278, 1524, 6730 bp. The MUC1-E1A adenoviral clone was designated CN226 (ref. 39.112).

MUC1 E1B Ad5 Plasmid (CN292)

An adenovirus vector in which the expression of the E1B gene is under control of the MUC1-TRE was constructed as follows.

The plasmid CN237 (MUC1 E1A/E1B, described above) was digested with EagI to excise the MUC1-TRE EagI fragment. The MUC1-TRE fragment was isolated by gel electrophoresis (1.2 % SeaKem Agarose) onto DEAE filter paper and ligated into CN124 (described above) which had been linearized with EagI. The resulting clone was confirmed by restriction digest: PinAI, 1924 and 7826 bp; HindIII/PinAI 807, 1199, 1924, 6730 bp. The MUC1 E1B clone has been designated CN292 (ref. 46.050).

Construction of MUC1-TRE E1A/E1B construct CN237

To construct adenoviral vector CN237, in which a MUC1-TRE controls expression of both E1A and E1B, a second MUC1-TRE was inserted upstream of the E1B gene in construct CN226, which already contained a MUC1-TRE controlling expression of E1A.

In more detail, a fragment containing the MUC1-TRE with EagI ends was obtained by PCR of **CN226** with the following primer pairs: 5' TAA CGG CCG CGG TGA CCA CTA GAG 3' (39.120A, upper primer-SEQ ID NO:64) and 5' TAT CGG CCG GCA GAA CAG ATT CAG 3' (39.120B, lower primer-SEQ ID NO:65). Amplification of the MUC1-TRE containing EagI ends utilized an annealing temperature of 55°C (30 cycles) with an extension temperature and time of 72°C for 60 seconds. PCR products were purified with the QIAQuick Spin PCR Purification Kit (Qiagen). The MUC1-TRE PCR product was digested with EagI and ligated in front of the E1B region of CN226, which had been linearized with EagI, which cuts just upstream of the E1B coding segment. The resulting clone was confirmed by

restriction digest: PinAI, 1997 and 9453 bp; SmaI, 179, 980, 1917, 2711, 6562 bp. The MUC1 E1A/E1B clone has been designated CN237 (ref. 39.143).

Homologous Recombination of CN226, CN237, and CN292 with BHG10 or BHG11

Adenovirus containing the MUC1-TRE regulating expression of E1A, E1B, and E1A/E1B, in the context of the otherwise intact genome have been obtained through homologous recombination with the right hand end plasmids BHG10 and/or BHG11 [Bett. et al. (1994); Microbix Biosystems Inc., Toronto] in 293 cells (human embryonic kidney cell line). The plasmids (e.g. CN226 and BHG10; or CN237 and BHG10, etc.) have been co-transfected into 293 cells via cationic lipids (DOTAP:DOPE™ 1/1 mole ratio) by a standard transfection protocol, including, but not limited to, that detailed below.

Adenoviral vector CN735, in which E1A is under control of MUC1-TRE, was obtained by homologous recombination of CN226 and BHG10. An adenoviral vector, to be designated CN744, comprising E1B under control of MUC1-TRE, can be obtained by homologous recombination of CN292 and BHG11. Adenoviral vector CN736, in which both E1A and E1B are under control of MUC1-TRE, was obtained by homologous recombination of CN237 and BHG10. These plasmids and adenoviral vectors are diagrammed in Figure 9.

EXAMPLE 6

Construction of Replication-Competent Adenoviral Vectors in Which Adenoviral Genes are Under Transcriptional Control of Probasin TRE

Adenoviral vectors in which a PB-TRE was placed upstream of E1A and/or E1B were constructed.

The probasin transcriptional response element (PB-TRE)

The 454 nucleotide fragment (nt about -426 to about +28) of the rat PB-TRE, which contains two androgen response elements (ARE sites), a CAAT box and a TATAA box (Figure 10, SEQ ID NO:9), was amplified by polymerase chain reaction (PCR) using rat genomic DNA as template and the synthetic oligonucleotides:

42.2.1 (SEQ ID NO:66):
5'-GATCACCGGTAAGCTTCCACAAGTGCATTTAGCC-3',
PinAI site underlined,
and
42.2.2 (SEQ ID NO:67):
5'-GATCACCGGTCTGTAGGTATCTGGACCTCACTG-3'
or oligonucleotides
42.2.3 (SEQ ID NO:68):
5'-GATCCGGCCGAAGCTTCCACAAGTGCATTTAGCC-3',
EagI site underlined,
and
42.2.4 (SEQ ID NO:69):
5'-GATCCGGCCGCTGTAGGTATCTGGACCTCACTG-3'.

The oligonucleotides created a unique PinAI (AgeI) site (A/CCGGT) or EagI site (C/GGCCG) at both ends of the PCR fragments. The PCR fragments were ligated into the pGEM-T vector (Promega) to generate plasmids CN249 and CN250. Similarly, CN256 was created using the same strategy but the PB-TRE fragment was ligated into the pCRT vector (Invitrogen); CN271 is identical to CN250 but with a HindIII site at the 5'-end. These plasmids provide the PB-TRE DNA fragments for the constructs reported below. In some of the adenovirus vectors described below, the endogenous (adenoviral) TREs were not deleted; rather, in each construct, the PB-

TRE was inserted between the endogenous TRE (e.g., the E1A TRE) and its respective coding segment (e.g., the E1A coding segment). In other vectors, the endogenous (Ad5) promoter-enhancer has been deleted, and the prostate-specific promoter-enhancer placed immediately upstream of an early gene.

PB-TRE-driven E1A Ad5 plasmid (CN251)

An adenovirus vector in which expression of an early gene, E1A, is under control of PB-TRE was constructed as follows.

CN124 is a derivative of construct pXC.1, which contains the wild-type left-hand end of Ad5, from nt 22 to 5790, including both E1A and E1B (McKinnon (1982) *Gene* 19:33-42). CN124 also has, among other alterations, an artificial PinAI site at Ad5 nt 547 (between the E1A transcriptional start at nt 498 and the E1A coding segment beginning with ATG at 560).

To construct CN124 from pXC.1, we introduced an AgeI site 12 bp 5' to the E1A initiation codon (Ad5 547) by oligonucleotide-directed mutagenesis and linked PCR. To achieve this, pXC.1 was PCR-amplified using primers:

5'-TCGTCTTCAAGAATTCTCA (15.133A) (SEQ ID NO:14), containing an EcoRI site, and

5'-TTTCAGTCACCGGTGTCGGA (15.134B) (SEQ ID NO:15), containing an extra A to introduce an AgeI site. This created a segment from the EcoRI site in the pBR322 backbone to Ad5 560. A second segment of pXC.1 from Ad 541 to the XbaI site at Ad nucleotide 1339 was amplified using primers:

5'-GCATTCTCTAGACACAGGTG (15.133B) (SEQ ID NO:16) containing an XbaI site, and

5'-TCCGACACCGGTGACTGAAA (15.134A) (SEQ ID NO:17), containing an extra T to introduce an AgeI site. These two PCR-amplified DNA segments were mixed and amplified with primers 15.133A and 15.133B to create a DNA segment from the EcoRI site to the XbaI site of pXC.1. This DNA segment encompasses the

leftmost 1317 bases of Ad sequence and contains an AgeI site at Ad 547. This DNA segment was used to replace the corresponding segment of pXC.1 to create CN95.

An EagI site was created upstream of the E1B start site by inserting a G residue at Ad5 1682 by oligonucleotide directed mutagenesis as above. To simplify insertion of an PB-TRE in the EagI site, the endogenous EagI site in CN95 was removed by digestion with EagI, treatment with mung bean nuclease, and re-ligation to construct CN114. The primers:

5'-TCGTCTTCAAGAATTCTCA (15.133A) (SEQ ID NO:14), containing an EcoRI site, and

5'-GCCCACGGCCGCATTATATAC (9.4) (SEQ ID NO:46), containing an EagI site, and

5'-GTATATAATGCGGCCGTGGGC (9.3) (SEQ ID NO:47) containing an extra G and an EagI site, and

5'-CCAGAAAATCCAGCAGGTACC (24.020) (SEQ ID NO:30), containing a KpnI site, were used to amplify the segment between 1682 and the KpnI site at Ad5 2048. Co-amplification of the two segments with primers 15.133A and 24.020 yielded a fragment with an EagI site at Ad5 1682 which was used to replace the corresponding EcoRI/KpnI site in pXC.1 to construct CN124.

CN124 was linearized with PinAI and dephosphorylated with calf intestinal alkaline phosphatase (New England Biolabs). CN249 was digested with PinAI to free the PB-TRE fragment. The PB-TRE fragment was then ligated into the PinAI-linearized CN124, producing CN251. CN253 is similar to CN251 except for the PB-TRE fragment is in the reverse orientation.

Thus, construct CN251 contains the PB-TRE inserted upstream of and operably linked to the E1A coding segment in the Adenovirus 5 genome.

PB-TRE-driven E1B Ad5 plasmid (CN254)

An adenovirus derivative in which the expression of the E1B gene is under control of the PB-TRE was constructed as follows.

CN124, described above, also contains an artificial EagI site at Ad5 nt 1682, or just upstream of the E1B coding segment. The PB-TRE fragment was excised from CN250 with EagI and inserted into CN124 digested with EagI. This produced CN254, which contains the PB-TRE immediately upstream of and operably linked to the E1B coding segment.

CN255 is identical to CN254, but the orientation of the PB-TRE insert is reversed.

CN275 is the same as CN254, but with a HindIII site at the 5'-end.

PB-TRE-driven E1A and PB-TRE-driven E1B Ad5 plasmid (CN268)

An adenovirus vector in which expression of both E1A and E1B are driven by PB-TRE was constructed as follows.

CN251, described above, comprises a PB-TRE fragment inserted just upstream of the E1A coding segment.

CN268 was generated by inserting a second PB-TRE in front of the E1B gene in CN251. A PB-TRE fragment was excised from CN250 by EagI-digestion and ligated into EagI-digested CN251 to create CN268. The final construct is a plasmid with PB-TRE driving E1A and a second PB-TRE driving E1B. CN269 is the same as CN268 but the orientation of the second PB-TRE is reversed. Constructs CN251, CN254, and CN268 are shown schematically in Figure 11.

EXAMPLE 7

Construction of Replication-Competent Adenoviral Vectors in Which Adenoviral Genes are Under Transcriptional Control of a Kallikrein TRE

hKLK2 promoter-driven E1A Ad5 plasmid CN303

- CN303 was produced by inserting the minimal *hKLK2* promoter (-324 to +33) just upstream of the E1A coding segment in a derivative of pXC-1, a

plasmid containing the left hand end of the Ad5 genome.

- CN124 is a derivative of construct pXC-1 which contains the wild-type left hand end of Ad5, including both E1A and E1B (McKinnon (1982) *Gene* 19:33-42). CN124 also has among other alterations, an artificial PinAI site at Ad5 nt 547 (just upstream of the E1A transcriptional start at nt 560 and the E1A coding segment beginning with ATG at 610). CN124 was linearized with PinAI and dephosphorylated with calf intestinal alkaline phosphatase (New England Biolab).

- CN294 was digested with PinAI to free the *hKLK2* promoter. The *hKLK2* promoter was then ligated into the PinAI linearized CN124, producing CN303. CN304 is similar to CN303 except for the *hKLK2* promoter fragment is in the reverse orientation.

- CN421 was constructed by inserting an *hKLK2*-TRE (comprising an *hKLK2* enhancer from nucleotides -5155 to -3387 relative to the *hKLK2* gene transcription start site (nucleotides 6859 to 8627 of SEQ ID NO:1) and an *hKLK2* minimal promoter as in CN379; see Table 1 and Figure 16) into CN306. The *hKLK2*-TRE fragment was amplified by PCR from CN379, digested with PinAI and ligated into similarly cut CN306, to produce CN421.

- CN438 was constructed by inserting an *hKLK2*-TRE (comprising an *hKLK2* enhancer from nucleotides -4814 to -3643 relative to the *hKLK2* gene transcription start site (nucleotides 7200 to 8371 of SEQ ID NO:1) and a minimal *hKLK2* promoter as in CN390; see Table 1 and Figure 16) into CN306. The enhancer fragment was amplified by PCR from CN390, digested with PinAI and ligated into similarly cut CN306, to produce CN438.

- CN306 was derived from CN124 by removing the endogenous 64-nucleotide E1A promoter.

- CN124 is a derivative of construct pXC-1 which contains the wild-type left hand end of Ad5, including both E1A and E1B (McKinnon (1982) *Gene* 19:33-42). CN124 also has among other alterations, an artificial PinAI site at Ad5 nt 547 (just upstream of the E1A transcriptional start at nt 560 and the E1A coding segment beginning with ATG at 610). CN124 was linearized with PinAI and dephosphorylated with calf intestinal alkaline phosphatase (New England Biolabs).

- CN390 was constructed as follows. A fragment with KpnI and XhoI sites at the ends was amplified from CN379 with synthetic oligonucleotides 51.96.3 (5'-GAT CGG TAC CAA AAG CTT AGA GAT GAC CTC CC-3'; SEQ ID NO:70) and 51.96.4 (5'-GAT CCT CGA GGC AAT AAT ACC GTT TTC TTT TCT GG-3'; SEQ ID NO:71). The resulting fragment was digested with XhoI and KpnI, then cloned into similarly cut CN325, to generate CN390. CN390 has a 1.17-kb hKLK2 enhancer (nucleotides 7200 to 8371 of SEQ ID NO:x) and a minimal hKLK2 promoter (-324 to +33 relative to the transcription start site).

- CN379 has, in addition to the minimal *hKLK2* promoter, the *hKLK2* 5' flanking region from -5155 to -3387 (nucleotides 6859 to 8627 of SEQ ID NO:1) driving expression of the luciferase gene.

Construction of adenovirus vectors comprising hKLK2-TRE controlling expression of adenovirus E1A

- CN749, comprising an hKLK2 promoter (-324 to +33) driving adenovirus E1A gene expression, was generated by co-transfecting CN303 and pBHG10 into 293 cells.

- CN763, comprising an hKLK2-TRE promoter/enhancer from CN379 controlling transcription of E1A, was generated from CN421 and pBHG10. To produce plasmid CN421, the hKLK2-TRE was amplified from CN379 and cloned into CN306. Therefore, CN763 is an adenoviral vector in which an hKLK2-TRE comprising a minimal *hKLK2* promoter and the *hKLK2* 5' flanking region from -5155 to -3387 (nucleotides 6859 to 8627 of SEQ ID NO:1) controls expression of E1A.

- CN768, comprising an hKLK2-TRE controlling transcription of E1A, was constructed from CN438 and pBHG10. Thus, CN768 is an adenoviral vector in which an *hKLK2*-TRE (comprising an hKLK2 enhancer from nucleotides -4814 to -3643 relative to the *hKLK2* gene transcription start site (nucleotides 7200 to 8371 of SEQ ID NO:1) and a minimal *hKLK2* promoter controls expression of E1A.

In vitro characterization of adenoviral constructs comprising an adenoviral gene under transcriptional control of an hKLK2-TRE

Plaque assays

To determine whether the adenoviral constructs described above replicate preferentially in prostate cells, plaque assays were performed. Plaquing efficiency was evaluated in the following cell types: prostate tumor cell lines (LNCaP), breast normal cell line (HBL-100), ovarian tumor cell line (OVCAR-3, SK-OV-3), and human embryonic kidney cells (293). LNCaP cells express both androgen receptor and PSA, while the other cell lines tested do not. 293 cells serve as a positive control for plaquing efficiency, since this cell line expresses Ad5 E1A and E1B proteins. The plaque assay was performed as follows: Confluent cell monolayers were seeded in 6-well dishes eighteen hours before infection. The monolayers were infected with 10-fold serial dilutions of each virus. After infecting monolayers for four hours in serum-free media (MEM), the media was removed and replaced with a solution of 0.75% low melting point agarose and tissue culture media. Plaques were scored two weeks after infection. CN702 has no modifications in its E1 region and is used as a wild type control. CN706 demonstrates selective cytotoxicity toward PSA-expressing cells *in vitro* and *in vivo*. Rodriguez et al. (1997) *Cancer Res.* 57:2559-2563.

Table 12:

	293	**LNCaP**	**HBL-100**	**OVCAR-3**
Viruses				
CN702	**100**	**100**	**100**	**100**
CN706	**100**	**23**	**2.4**	**5.5**
CN763	**100**	**35**	**1.2**	**1.9**
CN768	**100**	**29**	**1.3**	**3.9**

Table 12 shows the results of plaque assays performed with the adenoviral vectors described above. The results are expressed as percent of wild-type adenovirus plaque-forming units (PFU) per ml. The average titer of duplicate samples for the viruses tested. The titer for a particular virus in all cell lines was normalized to its titer on 293 cells. Once the titers on a cell type were normalized to 293 cells, the normalized numbers of the recombinant viruses were compared to CN702. A ratio of less than 100 suggests that the virus tested plaques less efficiently than CN702. Conversely, a ratio greater than 100 suggests that the virus plaques more efficiently than CN702.

The following observations were made. First, *hKLK2*-TRE engineered adenoviruses demonstrate preferential replication in prostate tumor cells. Since this carcinoma expresses androgen receptors, the *hKLK*-TRE contained in the adenoviral vectors should be active in promoting the transcription of the adenovirus early genes. The data presented in Table 12 suggest that the *hKLK2*-TRE containing adenoviral vectors induce cytopathic effects with a lower efficiency than wild type adenovirus in prostate tumor cells. Second, *hKLK2*-TRE controlled adenoviruses show a dramatically lower plaquing efficiency in non-prostate tumor cells when compared to wild type. For example, in the ovarian carcinoma cell line OVCAR-3, CN763 and CN768 produced about 25 to 50-fold less plaques than wild type Ad5. The results are similar for these two viruses in HBL-100 cells, where virus replication is also severely compromised. Third, PSA-TRE adenoviral vectors and *hKLK2*-TRE adenoviral vectors give similar plaques in HBL-100 and OVCAR-3 cells. Thus, like PSA-TRE adenoviral vector CN706, *hKLK2*-TRE adenoviral vectors were significantly attenuated relative to wild-type adenovirus in non-prostate cells, but these vectors grew comparably in prostate tumor cells.

It is evident from the above results that adenoviruses can be provided as vehicles specific for particular host cells, where the viruses are replication-competent.

The viruses may be vehicles for a wide variety of genes for introduction in the target host cells. Particularly, employing the prostate specific element, the early genes essential for replication may be modified so as to be under the control of prostate cell responsive elements.

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.